Soil carbon cycling responses to elevated CO_2 and nitrogen addition

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Sarah E. Hobbie and Dr. Peter B. Reich

October 2017

Acknowledgements

There are many people who supported me throughout my PhD, contributed to my intellectual growth, and the development of the work and ideas presented here.

I would like to thank my advisors, Sarah Hobbie and Peter Reich, for their incredible support – both of me as a person, as well as my development as a scientist. Their dual commitment to conducting rigorous, impactful research and leading rich, engaging lives make them such role models. My committee members, Dave Tilman and Nancy Johnson, contributed their distinct perspectives, which enriched my own. I would also like to thank Jess Gutknecht for her microbial insights and support throughout the process.

I am thankful for everyone in the Ecology, Evolution, and Behavior (EEB) Department who actively foster its supportive and collaborative environment. There are many people I learned from over the years, but I would like to specifically thank Bob Sterner, Elizabeth Borer, Eric Seabloom, Forest Isbell, Jacques Finlay, and Scott Lanyon for so many helpful discussions on everything from ecological theory to advocating for one's whole self to building lasting institutional culture. I am also so thankful for Lisa Wiggins and all she has done in her time in EEB to support graduate students.

There were several groups I participated in that fostered discussion of topics in ecology and environmental leadership that benefited this work and my own growth. Specifically, I am grateful for being a part of the Hobbie Lab Group, the Forest Ecology Lab Group, the

UMN Ecosystem Group, the Midwest Aggregate Group, the UMN Institute on the Environment's Boreas Leadership Program, and my EEB Cohort.

There are many individuals who keep the experiments and labs running, and whose expertise in various methods and the Cedar Creek Ecosystem Science Reserve made them invaluable resources. Specifically, I would like to thank Chris Buyarski, Cindy Buschena, Kally Worm, Troy Mielke, and Susan Barrott. I also benefited from working with many undergraduate students and interns who helped get the work done, allowed me to discover the joys of mentoring, and inspired me with their stories and excitement.

This research was made possible by funding from the National Science Foundation (NSF) and the University of Minnesota (UMN) Graduate School, College of Biological Sciences and EEB Department. Specific grants and fellowships include: the Cedar Creek Long Term Ecological Research Program (NSF DEB-9411972, DEB-0080302, DEB-0620652, and DEB-1234162), a CAREER Award (NSF DEB-0347103) a Graduate Research Fellowship (NSF 00039202), a Doctoral Dissertation Improvement Grant (NSF 1501769), the Inter-university Training in Continental-scale Ecology Fellowship (NSF 1137336), the UMN Graduate Excellence Fellowship, Carolyn Crosby Grant, Carol and Wayne Pletcher Fellowship, Florence Rothman Fellowship, and EEB Research and Travel Grants.

I am so grateful for the wonderful friends who supported me and without whom graduate school would not have been nearly as fun or enriching. I especially want to thank Amy Kendig, Anika Bratt, Maria Rebolleda-Gomez, Marta Lyons, Kyle Gill, Jake Grossman, Jared Rubenstein, Charlotte Riggs, Jane Cowles, Salli Dymond, Christine O'Connell, Meredith Steck, Anna Baker, Amanda Gorton, Danielle Draebeck, Cristy Portales, Gabi Huelgas Morales, Liana Burghardt, Eric Lind, and Habacuc Flores-Moreno.

I cannot thank my family enough. I have many extended family members who encouraged my curiosity and whose work in environmental science and conservation fueled my own interests. Mostly, though, I want to thank my parents, Madelyn Leopold and Claude Kazanski, and my brother, Topher Kazanski, for all their support and faith in me through the process.

Finally, I want to thank my partner, Nolan O'Brien, for all that he's done to support and encourage me in this endeavor.

Dedication

This thesis is dedicated to two women whose love of place inspired their pioneering contributions to science and conservation, as well as my first interest in prairies: my great aunts, Nina and Estella.

Abstract

Soils are the largest terrestrial pool of carbon (C). Understanding the mechanisms responsible for C loss from soils, and how they might respond to global change, is therefore necessary for predicting future C-climate feedbacks. However, it remains uncertain how soil C processes will respond to an increasingly changing global environment, marked by rising carbon dioxide (CO_2) emissions and nitrogen (N) deposition from human activities. While decades of experiments have provided insight into how inputs of organic matter to soils might respond to elevated CO_2 and N deposition, there is still considerable uncertainty around how global change will affect losses of C from soils. Therefore, this dissertation assesses the effects of elevated CO_2 and N addition on three distinct C cycling processes that collectively contribute to the net release of C from soils: 1) soil aggregation, a key control of soil organic matter (SOM) accessibility to decomposers, 2) microbial decomposition of SOM in bulk soil, and 3) priming of SOM decomposition by roots and their associated microbial communities. To do this, we developed a suite of field- and lab-based experiments that built on the frameworks of two long-term global change experiments at the Cedar Creek Ecosystem Science Reserve in central Minnesota. Overall, we found that global change could result in distinct responses in specific soil C cycling that could counteract each other. Specifically, elevated CO_2 increased aggregation, hence *reducing accessibility of SOM* to decomposers, likely due to both bacterial and fungal activity. C loss from microbial respiration in the absence of roots consistently did not respond to long-term N addition, across different ecosystem types, which contradicts current thinking that N addition may

inhibit microbial decomposition and lead to greater accumulation of C in soils. Finally, soil C loss from microbial decomposition in the rhizosphere increased, on average by 34-39%, with elevated CO_2, likely due to increased C inputs to rhizosphere soils, whereas it decreased by 29%, on average, with N addition. Collectively, these findings highlight the importance of assessing multiple processes at play in soil C cycling, as individual mechanisms might not reveal the actual response in total soil C loss.

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INTRODUCTION

Soils are the largest terrestrial pool of carbon (C) (Ciais *et al.*, 2013). Proportionally small changes in soil C could therefore have large consequences for atmospheric CO_2 levels. However, how a changing global environment, marked by increasing concentrations of atmospheric CO_2 (Friedlingstein *et al.*, 2014) and nitrogen (N) deposition (Galloway *et al.*, 2008), will affect soil C stocks in the future remains uncertain. Understanding the mechanisms responsible for retention and loss of C from soils, and how those mechanisms might respond to global change, is necessary for predicting future climate-carbon feedbacks.

Ultimately, the amount of C held in soils depends on the balance between *inputs* from new biomass growth and allocation belowground and *outputs* from decomposition. Although how long the effects of elevated CO_2 and N addition will be sustained in ecosystems is not entirely clear, we do have a generally good understanding of how these global changes will affect biomass growth and allocation of C. Studies of grasslands and forests across the globe show elevated CO_2 tends to stimulate plant biomass growth across many studies globally (Norby *et al.*, 2005; Lee *et al.*, 2010). Its positive effect will likely depend on the relative availability of other critical plant resources like N and water (Reich *et al.*, 2006; Norby *et al.*, 2010; Reich & Hobbie, 2013; Reich *et al.*, 2014), and may be enhanced at higher levels of plant species diversity (Reich *et al.*, 2001; 2004). Elevated CO_2 also results in increased C allocation belowground from increased root biomass, but also greater release of C through rhizodeposition (Adair *et al.*, 2009). N

addition also generally increases biomass growth (LeBauer & Treseder, 2008; Fay *et al.*, 2015) and allocation of C belowground through enhanced root biomass (Adair *et al.*, 2009; Fornara & Tilman, 2012).

In contrast, there is much more uncertainty around the effects of elevated CO_2 and N addition on the other side of the equation, *outputs*. To date, experiments assessing effects of elevated CO_2 on loss of C from soils, have generally found increases, although the magnitude of the response can vary substantially (Pendall *et al.*, 2003; Bernhardt *et al.*, 2006; Adair *et al.*, 2011; van Groenigen *et al.*, 2014). The effects of N addition on C loss have been more mixed: meta-analyses have found soil respiration to decrease with N addition in temperate forests (Janssens *et al.*, 2010), increase in grasslands (Zhou *et al.*, 2014), and also not change (Liu & Greaver, 2010). Soil respiration combines multiple processes, however, and global change factors may differentially affect each.

In order to predict global change effects on total loss of soil C therefore requires more detailed mechanistic testing of the multiple processes that might be at play. Specifically, an emerging paradigm considers *accessibility* of soil organic matter (SOM) to be a primary determinant of whether soil C remains stored in soil or gets decomposed and released back to the atmosphere as CO_2 (Dungait *et al.*, 2012; Schimel & Schaeffer, 2012). This view reflects a growing abandonment of the long-held belief that C residence time in soils is linked to some inherent "recalcitrance" based on its chemical composition (Kleber *et al.*, 2011; Schmidt *et al.*, 2011; Dungait *et al.*, 2012). Instead, this new

framework first identifies how much of total soil C is accessible to be decomposed, and then focuses on the environmental and biological factors influencing the rate of decomposition of that accessible C. To predict future net retention of C in soils under global change, it is thus critical to assess impacts on both 1) *accessibility* of SOM and 2) *decomposition* of accessible SOM.

The overarching goal of this dissertation is to assess the effects of elevated CO_2 and N addition on three key C cycling processes that collectively contribute to the net release of C from soils. We do this by specifically addressing the following questions:

1. How do soil microbes influence aggregation, a key mechanism for protection of soil organic matter from decomposition, or reduced *accessibility,* under elevated CO_2 and N addition (*Chapter 1*)?
2. How does long-term N addition affect microbial respiration of soil organic matter, and do the responses differ by ecosystem type (*Chapter 2*)?
3. What are the effects of elevated CO_2 and N addition on rhizosphere priming (or root-induced decomposition of soil organic matter by rhizosphere microbes), and do the effects differ in soils under different plant species (*Chapter 3*)?

Combined, this dissertation employs creative experimental approaches to test critical questions related to soil C cycling and how it might respond to future environmental change. The findings we report offer insight into how the largest terrestrial pool of C may change in the future.

CHAPTER 1

Microbially mediated short-term increases in soil aggregation with elevated CO_2

Abstract

Soil aggregation is important for soil structure and carbon (C) protection. Soil microbes can affect aggregation and are sensitive to environmental changes. For instance, elevated CO_2 has been shown to increase soil aggregation, possibly due to increased activity or growth of filamentous fungi whose hyphae can enmesh organic matter and soil particles, facilitating aggregation. However the specific mechanism by which elevated CO_2 increases aggregation has not been shown. We designed an experiment to test whether the presence of different microbial groups (bacteria versus fungi) mediates the effects of elevated CO_2 on soil aggregation. We deployed microbial in-growth bags in a factorial CO_2 (ambient+180ppm) by nitrogen (N) addition (+4g N m^{-2} y^{-1}) sub-experiment of the Biodiversity, CO_2, and Nitrogen (BioCON) grassland experiment in sandy soils at the Cedar Creek Ecosystem Science Reserve in Minnesota, USA. Mesh bags were constructed to allow access to only bacteria (1μm) or to bacteria and fungi (28μm), were filled with sterilized, sieved soil, and harvested after one and two growing seasons. We measured aggregation using optimal-moisture sieving (target moisture of 10%, as described by Bach & Hofmockel, 2014). Total aggregation (as the mean-weighted diameter, per van Bavel, 1950) increased with elevated CO_2 after the first growing season in both bags where fungi were included and excluded, due to an increase in the proportion of medium macroaggregates (1-2mm). Overall, soils after the first growing

season were also more aggregated when fungi were included compared to when they were excluded, again driven by differences in medium macroaggregates. However, treatment effects were absent after the second growing season. Our findings support the idea that microbes may help determine soil structure in sandy soils under future environmental conditions and further suggest that under elevated CO_2, increases in aggregation could come from both bacteria and fungi. Our work also highlights how dynamic soil aggregate formation is, however, and that microbially mediated aggregation can respond quickly to environmental conditions, which has implications for future soil carbon cycling under elevated CO_2.

Introduction

The formation of soil aggregates is a key mechanism by which soil carbon (C) is physically protected from decomposition (Six *et al.*, 2002; Bronick & Lal, 2005; Verchot *et al.*, 2011). C-rich organic matter and mineral particles bind together or get trapped by roots or fungal hyphae to form aggregates, rendering organic matter that is less accessible to microbes, and thus protected from microbial decomposition (Miller & Jastrow, 1990; Jastrow *et al.*, 1998; Six *et al.*, 2000). This physical protection of organic matter through aggregation can result in C storage on decadal timescales (Trumbore, 1997). Although aggregation is highly correlated with clay content in soils because high-surface area and charged clays tend to adsorb organic matter onto mineral surfaces (Tisdall & Oades, 1982), plant roots and soil microbes, particularly fungi (Miller & Jastrow, 1990; Tisdall *et al.*, 1997), also facilitate aggregation and may be particularly important in determining aggregation in low-clay systems (Oades, 1993).

Because plant roots and soil microbes can be sensitive to increasing atmospheric CO_2 concentrations, aggregate formation and protection of C could also respond to rising CO_2 in the future. Under elevated CO_2 plants invest more C belowground (Adair *et al.*, 2009) and increase root exudation or allocation of C to fungal symbionts (Sanders *et al.*, 1998), hence the often observed CO_2-induced stimulation of stimulating microbial growth and activity (Treseder, 2004; Drigo *et al.*, 2008). Relatedly, since nitrogen (N) deposition can also influence microbial communities (Treseder, 2004; 2008), there could also be cascading effects on soil aggregation and protection of C. Depending on the direction of

response, changes in aggregation under elevated CO_2 and N addition could slow or accelerate the release of CO_2 back to the atmosphere. In grasslands experimentally exposed to elevated CO_2, soil aggregation has increased (Rillig *et al.*, 1999a; 2001), decreased (Niklaus *et al.*, 2003; 2007), or not changed (Eviner & Chapin, 2002). Whether aggregation responds to CO_2 could depend on soil type and whether plant-induced changes in soil moisture substantially influence wetting-drying cycles, which can increase aggregate formation, particularly in moderately weathered soils containing more clay minerals (Denef *et al.*, 2002).

However, shifts in abundance of arbuscular mycorrhizal fungi and other filamentous soil fungi at higher levels of atmospheric CO_2 or under N addition could be particularly important for soil aggregation (Rillig & Mummey, 2006). These fungi's branching hyphal structure is especially effective at enmeshing soil particles and organic matter into macroaggregates (>250μm diameter) (Miller & Jastrow, 1990; Tisdall *et al.*, 1997). For example, in a long-term field study, arbuscular mycorrhizal abundance was found to be the best determinant of soil aggregation (Wilson *et al.*, 2009). Furthermore, as plants invest more resources belowground under elevated CO_2, soil fungal:bacterial ratios could increase (Chung *et al.*, 2007), and arbuscular mycorrhizal fungi will likely become more abundant (Rillig *et al.*, 1999b; Treseder, 2004; Antoninka *et al.*, 2011), which could lead to increased aggregation. Indeed, in an experiment in two California grasslands, Rillig *et al.* (1999) found an increase in macroaggregates (1-2mm diameter) under elevated CO_2, which corresponded to greater glomalin production, a heat-stress protein produced by

arbuscular mycorrhizal fungi (Driver *et al.*, 2005; Purin & Rillig, 2007). However, whether CO_2 increases aggregation and protection of C via its effects on fungal processes or other mechanisms has not been directly tested. In contrast, arbuscular mycorrhizal abundance could decline under increased N addition (Johnson *et al.*, 2003; Treseder, 2004), potentially leading to less soil aggregation.

Although fungi, and arbuscular mycorrhizal fungi in particular, are commonly considered a primary driver of soil aggregation (Miller & Jastrow, 1990; Wilson *et al.*, 2009), bacteria can also play a role in aggregate stability. All microbes produce extracellular polymeric substances (EPS), like polysaccharides, proteins, and nucleic acids, that act as strong binding agents in biofilms (Wingender *et al.*, 1999). As a result, bacteria-produced EPS can help adhere soil particles and organic matter together and stabilize aggregates, particularly microaggregates (<250μm diameter) (Oades, 1993). In one study, for example, aggregate stability was greater when biomass of active bacteria was higher, and was unrelated to fungal hyphal length (Eviner & Chapin, 2002). Furthermore, like fungi, soil bacteria could increase in abundance and activity under elevated CO_2 (Drigo *et al.*, 2009) due to enhanced allocation of plant photosynthate belowground. Bacteria could also increase in abundance (Gutknecht *et al.*, 2012) or not change (Treseder, 2008) with greater N deposition, although this could depend on the relative availability of other nutrients. However, the relative contributions of bacteria and fungi to aggregation under elevated CO_2 and N addition remains largely untested. With increasing availability of data on how microbial communities respond to environmental change, insight into how

these broad groups affect soil aggregation would aid in predicting soil function responses to soil community changes.

Our objective was to test how microbes affect soil aggregation and C protection under elevated CO_2 and N enrichment. We tested this question by experimentally including and excluding fungi using an in-growth bag approach where bags with different mesh sizes were filled with sterilized, sieved soil and planted in the soil within a global change grassland experiment. We hypothesized that there would be more aggregation and protection of C at elevated CO_2 and less with N addition, but that effects of CO_2 would be greater than those of N given relatively greater plant C inputs belowground (Adair *et al.*, 2009), which could stimulate microbial activity and hence promote microbial release of binding products and increase hyphal growth (Antoninka *et al.*, 2011). Furthermore, we expected greater aggregation and protection of C in the larger-mesh bags where fungi could enter, because hyphal enmeshment of particles would increase aggregate formation more so than bacterial products.

Previous studies have compared observations of aggregate density to fungal abundance (Rillig *et al.*, 1999a; 2001), but few have experimentally manipulated fungal presence. Those that have did so using fungicide (Beare *et al.*, 1997; Wilson *et al.*, 2009), which takes away the living function of fungi but does not remove the physical hyphal networks already established. Since this physical network is a key component to the prediction that hyphae enmesh soil particles in aggregates, the fungicide treatment may not adequately

manipulate fungal presence. In contrast, we sought to directly test the effect of fungal presence by physically excluding fungi.

Materials and Methods

Study Site

This work was conducted in the Biodiversity, CO_2, and Nitrogen Experiment (BioCON), a long-term grassland global change study established in 1997 at the Cedar Creek Ecosystem Science Reserve in central Minnesota (Lat. 45N, Long. 93W; Reich *et al.*, 2001). The main BioCON experiment employs a split-plot, completely randomized design for the full factorial of treatments. Carbon dioxide is the whole-plot treatment (three ambient and three elevated rings) and N addition and plant species diversity are subplot treatments. The CO_2 treatment (+180ppm) is applied using Free Air CO_2 Enrichment and N is added (+4g N m^{-2} yr^{-1}) in the form of NH_4NO_3 three times over the growing season (May, June, and July). For this study we used the 48 16-species plots experiencing all CO_2 and N treatment combinations (ambient, +N, +CO_2, +N and +CO_2). The 16-species plots (which include four of each C4 grasses: *Andropogon gerardii, Bouteloua gracilis, Schizachyrium scoparium, Sorghastrum nutans*; C3 grasses: *Agropyron repens, Bromus inermis, Koeleria cristata, Poa pratensis*; legumes: *Amorpha canescens, Lespedeza capitata, Lupinus perennis, Petalostemum villosum*; and forbs: *Achillea millefolium, Anemone cylindrica, Asclepias tuberosa, Solidago rigida*) were used in order to hold constant plant species effects on aggregation, and because the species-rich plots have greater overall microbial biomass than plots with fewer species

(Chung *et al.*, 2007). Cedar Creek temperatures tend to range from daily summer highs of ~27°C to daily winter lows of ~4°C. Annual precipitation at Cedar Creek for the two years of the study was 679mm and 612mm, with most of the rainfall occurring during the growing season. The soils at BioCON (Argic Udipsamments) are nutrient poor, sandy outwash plain (93% sand, 3% silt, and 4% clay) (Dijkstra *et al.*, 2006a). However, despite their high sand content, there is evidence of aggregate formation (C. Adair, unpublished data).

Microbial Biomass Manipulation

We used an *in situ* approach to test the effects of fungal presence, N addition, and elevated CO_2 on soil aggregation. We installed microbial in-growth bags in the top 15cm of the soil profile within plots at BioCON (summer 2011) and harvested half after one growing season (October 2012) and the remaining half after two growing seasons (October 2013). Microbial in-growth bags were constructed using fine-mesh nylon to either exclude fungi and roots (1μm mesh; smaller-mesh) or allow fungi, but not roots, to penetrate (28μm mesh; larger-mesh) (Tisdall, 1991). Roots were excluded to focus specifically on the role of fungi compared to bacteria because prior work suggested fine roots contribute to soil aggregation by facilitating more fungal hyphae (Miller & Jastrow, 1990). Soil used in the bags was collected from the top 20cm of the soil profile from a site near BioCON (<0.3 km away), homogenized using a 2 mm sieve, and sterilized through two hour-long autoclave cycles at 121°C (spore tests (Mesa Labs, Omaha, NE, USA) confirmed sterilization was effective). Bags were 15cm x 15cm, and after being

filled with 250g of soil were approximately 1cm thick. Bags were inserted vertically into the soil at 20cm depth, using a flat shovel that allowed for creation of a small slit for the bag, and then covered.

Sampling

At the time of harvest, bags were gently removed from soil and placed in rigid plastic containers to decrease agitation during transport. Bags were transported to the lab in a cooler and then stored in the refrigerator (~4°C). Soil contents were emptied from the bags into their respective plastic containers, two subsamples were taken, and containers were returned to the refrigerator to dry slightly. One 10g subsample was processed immediately for gravimetric water content and another was returned to the container to track soil moisture change while in the refrigerator. When samples dried down to the "optimal moisture" content for the technique (~10-14%, see below), or if below 10%, soil was sieved, processed and measured for soil moisture at time of sieving, microbial biomass in the bag, aggregate fraction distributions, and C and N in each aggregate fraction. Soil moisture (at both the time of harvest and sieving) was determined by drying pre-weighed fresh soil at 105°C for at least 48 hours. Subsamples for microbial biomass were immediately frozen at -80°C.

Aggregate and Carbon Distribution

We assessed aggregate densities using the "optimal-moisture" sieving method (Mendes *et al.*, 1999; Schutter & Dick, 2002). We chose this method over the more commonly used

water-stable method (Six *et al.*, 2000) given the short time duration of the study and our interest in capturing biotic-induced changes in soil structure. Because the optimal-moisture sieving method applies less force than a water-stable method, it captures slightly less stable, but arguably equal or more biologically relevant, aggregate fractions as the force applied may be closer to what would be experienced in field conditions (Bach & Hofmockel, 2014). The procedure we used was reported in detail by Bach & Hofmockel (2014), and slightly modifies that of Schutter and Dick (2002). Briefly, once at their optimal moisture (~10-14%) for determining aggregate fractions (Schutter & Dick, 2002), soils were weighed and then shaken on a stack of sieves using a circular shaker (CSC Scientific Meinzer II™ Sieve Shaker, Fairfax VA, 22031) at 250 rpm for 3 minutes. The sieves used led to isolation of large macroaggregates (>2mm), medium macroaggregates (1-2mm), small macroaggregates (250μm-1mm), and microaggregates (<250μm). Contents from each sieve were collected and weighed to assess weight distributions (we averaged a 98.9% recovery rate). Individual fractions were air-dried, ground, and tested for C and N (Costech ECS 4010).

In addition to fraction weights, we also calculated a composite metric of aggregation for each bag. Mean-weighted diameter (*MWD*) was calculated using *Eq. 1* (Van Bavel, 1950), where P_{weight} is the proportion of the sample weight in that size fraction (e.g. >2mm). The coefficients are the median diameter size for each aggregate fraction, resulting in a higher *MWD* for samples with greater proportions in large and medium

macroaggregates. Aggregate MWD is used to assess how aggregated, overall, a sample is (Bach & Hofmockel, 2015).

(Eq. 1) $$MWD = 5*(P_{weight}>2mm) + 1.5*(P_{weight}>1mm) + 0.625*(P_{weight}>250\mu m) + 0.125*(P_{weight}<250\mu m)$$

Fungal and Bacterial Biomass

We assessed fungal, bacterial, and mycorrhizal biomass in order to test the effectiveness of the different mesh sizes. We did this using phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) analysis. Both bacteria and fungi have phospholipids in their cellular membranes, whereas only fungi produce neutral lipids as storage lipids, however in both cases specific lipids are produced by different types of bacteria and fungi so can serve as reliable indicators of biomass of those groups. We extracted, isolated, and quantified abundance of specific lipid biomarkers produced by certain fungi and bacteria following a modified Bligh and Dyer (1959) method described in detail by (Herman *et al.*, 2012). However, instead of discarding the NLFAs, we retained them and analyzed them separately to look at biomarkers specifically from general and arbuscular mycorrhial fungi (Olsson *et al.*, 1995; Schmidt *et al.*, 2017). Briefly, we extracted fatty acids from 2 g of freeze-dried soil using a chloroform extraction (6ml), with citrate buffer (5ml) and methanol (12ml). Phases were then left to separate overnight at room temperature, followed by removal of the aqueous phase by aspiration and reduction of the chloroform phase using a RapidVap evaporator. Lipid classes were separated using silica

column chromatography. We then converted extracted phospho and neutral lipids to fatty acid methyl esters using a milk alkaline methylation procedure (Schmidt *et al.* 2017), followed by extraction using 20µl of Hexane. Methyl-esterfied fatty acids were analyzed (2µl injection) on an Agilent 7890 Gas Chromatograph coupled to an Isoprime 100 Isotope Ratio Mass Spectrometer. We converted lipid peak areas to nmol lipid / g dry soil using a 13:0 internal standard added to each sample and also analyzed separately. We did this analysis for a subset of bags from the second harvest (2013; 32 total across all treatment combinations). Samples from 2012 were compromised, making PLFA and NLFA analyses impossible.

For our analysis we focused on key lipids as biomarkers of fungal and bacterial groups. We assessed biomarkers for total microbial biomass (sum of lipids from PLFA with greater than 10 and fewer than 20 carbons: 12:0, 13:0, 14:0, 15:0, 15:0 anteiso, 15:0 iso, 16:0, 16:0 10 me, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:0, 17:0 anteiso, 17:0 cyclo, 17:0 iso, 18:0, 18:0 10 me, 18:1ω9c, 18:1ω9t, 18:2ω6,9c, 19:0, 19:0 cyclo); *Glomus* arbuscular mycorhizal fungi (16:1ω5 phospho- and neutral lipids; (Graham *et al.*, 1995; Wilkinson *et al.*, 2002; Gutknecht *et al.*, 2012); *Gigaspora* mycorrhizae, common plant colonizers at Cedar Creek (Ji *et al.*, 2013), and non-mycorrhizal fungi (18:1ω9c phospho- and neutral lipids, (Graham *et al.*, 1995); general non-AMF fungi (18:2ω6,9c; Balser & Firestone, 2005; Gutknecht *et al.*, 2012); Gram positive bacteria (15:0 iso, Gutknecht *et al.* 2012, Wilkinson *et al.* 2002); and Gram negative bacteria (16:1ω7c, Gutknecht *et al.* 2012, Wilkinson *et al.* 2002).

Data Analysis

We evaluated short-term responses in aggregate *MWD*, relative aggregate fraction weights, and C content, after one and two growing seasons. For each of these responses, we used linear mixed-effects models to test the main effects of CO_2, nitrogen, and microbial in-growth bag type ("Bag" hereafter), and their interactions. We also included the gravimetric water content at the time samples were sieved in the model (and all two- and three-way interactions). We developed separate models for each year, after including it in the model and finding multiple by year interactions. We included treatments and all two- and three-way interactions as fixed effects and ring nested within CO_2 as a random effect. We used a maximum likelihood method of estimation. Models for all response variables (*MWD*, each relative fraction weight, and fraction C content) were checked to ensure model assumptions of normality and equal variance were met. When necessary, response variables were log-transformed, logit-transformed, or square root-transformed to meet model assumptions.

Finally, we used phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) analysis to assess effects of treatments and bag type on arbuscular mycorrhizal fungal, general fungal, and Gram positive and Gram negative bacterial biomass. We again used linear mixed effects models to test treatment effects on each of the key lipid biomarkers' abundance. Models included fixed effects of CO_2, N, and bag type, and all their two-way and three-way interactions, and a random effect of ring nested within CO_2. Again we

used the maximum likelihood method of estimation. We used R for all data analysis (R Core Team 2013). For the mixed effects models we used the lmer function within the lme4 package (Bates *et al.*, 2015) and the piecewiseSEM function (Lefcheck, 2015) for determining marginal and conditional R^2 values.

Results

Despite the relatively short duration of the study, and the sandy soils at Cedar Creek, we found significant changes in soil aggregation and C content with elevated CO_2 and in the different bag types. However, responses were not consistent over time.

Soil aggregation

The optimal moisture sieving method is sensitive to soil water content (Schutter & Dick 2002), which can affect how soil particles break apart with agitation, since drier soils can expose natural fractures or points of weakness (Dexter & Bird, 2001). Despite a target "optimal moisture" of ~10% (Mendes *et al.* 1999), soils were sieved along a range of moistures, from 0.8-12.8% because of different rates of drying. And indeed, aggregation was positively correlated with soil gravimetric water content (GWC) (Pearson's correlation $P<0.0001$, $r=0.64$). Bach & Hofmockel (2014) found very similar aggregate fraction distributions in a comparison between dry- and optimal-moisture sieving methods, which suggests that GWC lower than the ~10% target likely should not have led to distinct results. However, given its importance in sieving, we included GWC (as a covariate) at the time of sieving and its two- and three-way interactions with all fixed

effects in aggregation models. Here we focus on describing treatment effects, and when necessary how those effects were modified by soil moisture (i.e. when there were significant treatment*GWC interactions; Tables 1 and 2).

Soil aggregation responded to increased CO_2, but not consistently over time. Overall, as we hypothesized, soils under elevated CO_2 were more aggregated (MWD was higher) after the first growing season (CO_2 in 2012 P=0.0451, Fig. 1), due to having higher density of medium macroaggregates (1-2mm) (CO_2 P=0.0037, Table 1 and Fig. S1). There was also a significant CO_2*N*GWC interaction where aggregate MWD increased less steeply with GWC under elevated CO_2 and added N, compared to ambient N plots (CO_2*N*GWC P=0.0422, Fig. S2). However, in the second year those effects disappeared; elevated CO_2 had no effect on total aggregation, although it had some significant interaction effects with individual fractions (Table 1 and Fig. S1). However, there was a marginally significant CO_2*Bag interaction where aggregate MWD was slightly higher under elevated CO_2 but only in the smaller-mesh bags (CO_2*Bag treatment P=0.0520, Fig. S3). This response in *MWD* was likely driven by the similar response in medium macroaggregates (CO_2*Bag P=0.0285, Fig. S4). And although not captured in aggregate *MWD* response, there were some effects of N on individual fractions in the second year: the proportion of soil in large macroaggregates increased with N and the proportion in microaggregates decreased (P=0.0374 and P=0.0363, respectively, Fig. S1).

Aggregation also responded to the microbial in-growth bag type, but again not consistently over time. As hypothesized, aggregate *MWD* was higher in the larger-mesh bags, designed to include fungi, in the first year (Bag $P<0.0001$, Fig. 2), driven by increases in large macroaggregates (>2mm) ($P=0.0662$), and medium macroaggregates (1-2mm, $P=0.0015$). In the second year, the effect was not present (Table 1), although there was a marginally significant CO_2*Bag interaction effect, as described above (CO_2*Bag $P=0.0520$, Fig. S3).

Carbon content and distribution

Proportion of total C in aggregate fractions responded to increased CO_2, but as with aggregation, not consistently over time. Carbon content in fractions generally tracked with weight, with most C in the medium and small macroaggregate fractions (Fig. 3). In the first year, elevated CO_2 plots had slightly higher proportions of C in medium macroaggregates (2012 CO_2 $P=0.0277$, Table 2), and less C in small macroaggregates (2012 CO_2 $P=0.0161$, Table 2), than did ambient plots. This occurred despite a trend towards lower %C with elevated CO_2 in large-, medium-, and small macroaggregates in the first year (2012 CO_2 $P=0.0711$, $P=0.0690$, and $P=0.0547$, respectively, Table S1 and Fig. S5). However, in the second year elevated CO_2 had no effect on C distribution (Table 2), due to no change in aggregation with CO_2, as described above (Figs. 1 and S1) and no response in fraction %C (Table S1 and Fig S5).

Carbon content in aggregate fractions also responded to the in-growth bag treatment. In both years, soils in the larger-mesh bags where fungi were included showed slightly but significantly more C in the medium macroaggregate fraction (Bag 2012 P=0.0195 and 2013 P=0.0034, Table 2), and less C in the microaggregate fractions, although the effect in the second year was marginally significant (Bag 2012 P=0.0100 and 2013 P=0.0635, Table 2). Proportions of C in small macroaggregate fractions, however, were similar in the two different bag types in the first year (P>0.1, Table 2), but lower in the larger-mesh bags in the second year (Bag 2013 P=0.0318, Table 2). The proportion of C in the large macroaggregate fraction was not affected by bag type (P>0.1, Table 2). Again, C content patterns tracked closely with aggregate fraction weight distributions and often did not reflect differences in %C: in both years, %C trended lower in the larger-mesh bags across most fractions, although not always statistically significantly so (Table S1 and Fig. S6).

Microbial biomass responses to treatments in the second year

We measured phospholipid and neutral lipid fatty acids within in-growth bags to assess the effects of treatments, and the in-growth bags themselves, on bacterial, general fungal, and arbuscular mycorrhizal biomass. Unfortunately, we were only able to assess PLFA and NLFA from a subset of samples from the second year due to the first year's samples being compromised.

Overall, total microbial biomass, as the sum of lipids with between 10 and 20 carbons, was generally very low across all samples and did not respond much to treatments. Total

microbial biomass ranged from 0.05 nmol/g soil to 175.38 nmol/g soil and averaged 28.93nmol/g soil, which is about one-third of the average total biomass reported from PLFA measurements in 16-species plots in BioCON bulk soils in the past (although exact lipids included in calculations were slightly different; Chung *et al.* 2007). There was no change in total microbial biomass with elevated CO_2 (P>0.2) and no main effect of the bag treatment (P>0.4). There was, however, a significant N*Bag interaction (P=0.0152, Table 3A), such that total microbial biomass was higher in the small mesh bags, intended to only allow bacteria to enter, than in the larger-mesh bags under ambient N, but with added N microbial biomass was similar between the two bag types.

Abundance of biomarkers associated with arbuscular mycorrhizal fungi were not particularly sensitive to CO_2 and N treatments, and, to our surprise, did not generally differ between bag types (Table 3A). The 16:1ω5c phospholipid biomarker, which is most commonly associated with arbuscular mycorrhizal fungi (especially from the *Glomus* genera; (Graham *et al.*, 1995), but can also be produced by Gram negative bacteria, did not differ under elevated CO_2 or N addition (P>0.2). There was no effect of bag type on the 16:1ω5c phospholipid (P>0.7). Similarly, there were no main effects of CO_2, N, or bag type on abundance of the 16:1ω5c neutral lipid, an arbuscular mycorrhizal storage lipid (P>0.1, P>0.4, and P>0.8, respectively). There was also no bag effect on the relative abundance of the 16:1ω5c phospholipid (Bag P>0.2).

Another biomarker that is commonly produced by arbuscular mycorrhizal fungi in *Gigasporacea* (Graham *et al.*, 1995), which are common in Cedar Creek soil (Ji *et al.*, 2013), but is also produced broadly by other fungi, is the 18:1ω9c lipid. There was a significant interactive effect of N*Bag on 18:1ω9c from PLFA (P=0.03495, Table 3A): the effect was not very clear, but the smaller-mesh bags appeared to have slightly lower abundance of the 18:1ω9c PLFA with N addition than without N addition, whereas in the larger-mesh bags 18:1ω9c abundance was very similar in with and without N addition. The 18:1ω9c from NLFA did not differ across treatments (CO_2 P>0.6, Nitrogen P>0.5, Bag P>0.6). However, in terms of relative abundance, there was a marginally significant CO_2*Bag effect where relative abundance of the 18:1ω9c phospholipid was greater in larger-mesh bags than smaller-mesh bags, as expected, but only under ambient CO_2 (CO_2*Bag P=0.0991, Table 3B).

Abundance of the non-mycorrhizal, general fungal biomarker, 18:2ω6,9c from PLFA, did not differ with elevated CO_2, N addition, or bag type (P>0.4, P>0.2, and P=0.0839, respectively, Table 3A). There were also no differences across treatments in the absolute abundance of 18:2ω6,9c from NLFA (CO_2 P>0.7, Nitrogen P>0.9, Bag P>0.5). However, in terms of relative abundance, 18:2ω6,9c was more relatively abundant in the larger-mesh bags, intended to allow fungi to enter, but only under ambient CO_2 (under elevated CO_2, 18:2ω6,9c was similar between the two bag types; CO_2*Bag P=0.0426, Table 3B).

Bacterial biomarkers differed with treatments (Table 3A). The Gram positive phospholipid, 15:0 iso, and the Gram negative phospholipid, 16:1ω7c, showed similar responses to treatments: both had a significant N*Bag interaction (15:0 iso P=0.0142 and 16:1ω7c P=0.0194 such that their lipids were less abundant with N addition in the smaller-mesh bags and slightly trending to be more abundant with N addition in the larger-mesh bags. However, in terms of relative abundance, both the 15:0 iso and 16:1ω7c phospholipids had higher relative abundance in larger-mesh bags than the smaller-mesh bags (Bag P=0.0179 and P=0.0268, respectively).

Discussion

We found partial support for our hypothesis that elevated CO_2 would increase aggregation and protection of C, due to stimulation of microbial activity. Aggregate MWD was higher under elevated CO_2 after the first year, but not the second. However, in the second year we did not see microbial biomass, or individual biomarkers, greatly increase in abundance under elevated CO_2. We had also expected more aggregation and protection of C in the larger-mesh bags, where fungi could enter and we hypothesized hyphal enmeshment of particles would further increase aggregation. Again, we found only partial support of this hypothesis: aggregate MWD was greater in larger-mesh bags in the first year, but not statistically so in the second year. Furthermore, microbial biomass data from the second year showed no real difference in abundance of fungal biomarkers between the two bag types, indicating that at least in the second year of the experiment, the bags intended to exclude fungi (the smaller-mesh) did not do so. Here,

we discuss possible rationales for the aggregation response, difference between the years, and issues with the in-growth bag treatment.

Aggregation response to treatments

The increase in aggregation with elevated CO_2 in the first year could relate to increased microbial activity. Prior work from BioCON has shown that plant communities invest more C belowground under elevated CO_2 both to root biomass (Reich *et al.* 2001), but also independently of root biomass, likely as exudation and rhizodeposition (Dijkstra *et al.*, 2006a; Adair *et al.*, 2009). This increase in C availability could fuel both fungal and bacterial biomass growth and production of extracellular products. Although prior studies have suggested that elevated CO_2 mainly enhances aggregation by increasing fungal abundance, particularly AMF and their hyphae or glomalin (Rillig *et al.*, 1999a), it is also possible that increased abundance of bacterial products at elevated CO_2 (Finzi *et al.*, 2006) could stimulate aggregation. This is in keeping with other recent work that highlights the importance of microbial activity as stimulating release of products that can more easily stabilize onto mineral surfaces (*sensu* Cotrufo *et al.*, 2013; 2015). Perhaps, those same products can facilitate greater particle binding as well. Indeed, we found the CO_2-induced increases in aggregation across both in-growth bag types, suggesting that both bacteria as well as fungi could stimulate aggregation under elevated CO_2. However, the PLFA data from the second year of the experiment (when we observed no CO_2 effects on aggregation), did not suggest a general stimulation of microbial biomass with elevated CO_2 (Table 3; although, see below on in-growth bag treatments).

Elevated CO_2 also seems to have increased aggregation in part due to its impact on soil moisture, but not entirely so. Soils under elevated CO_2 can have higher soil moisture because plants reduce their stomatal conductance under elevated CO_2 (Lee *et al.*, 2011), resulting in lower transpiration and greater water retention in soils (Adair *et al.*, 2011). Higher soil moisture can in turn stimulate greater microbial biomass and activity (Brockett *et al.*, 2012), which could facilitate aggregation. In our experiment, field-level soil moisture in bags under elevated CO_2 tended to be higher than those experiencing ambient conditions (harvest soil GWC P=0.0007). However, CO_2-induced increases in soil moisture cannot explain the observed positive CO_2 effect on aggregation in the first year (Table 1 and Fig. 1) since CO_2 was still a significant term in the statistical model after including soil moisture. Furthermore, because soil moisture at time of sieving (included in the model) was moderately related to soil moisture at the time of harvest (R^2=0.47, P<0.001), the soil moisture included in models contained some legacy of field moisture differences (and hence the imbedded effect of elevated CO_2). Prior work at BioCON also found that elevated CO_2 led to increased belowground C cycling independently of its positive effects on soil moisture (Adair *et al.* 2011). In our study, in addition to the positive effects of CO_2 on soil moisture, an increase in available C substrates, from greater belowground C allocation, under elevated CO_2 (Adair *et al.*, 2011) may have stimulated the microbial community, leading to increases in aggregation, as described above.

Difference in response between years

The difference in aggregation response between the two years highlights the dynamic nature of aggregate formation, which we were able to capture with the optimal moisture sieving method. Compared to wet sieving, which isolates particularly stabile aggregates able to withstand maximum pressure gradients and slaking, the method we used captures stability of aggregates exposed to more field-relevant conditions (less pressure and disruption than the wet-sieving approach; Bach & Hofmockel 2014). The process of aggregate formation is dynamic, with aggregates forming and destabilizing, before forming again (Six *et al.*, 2004). For example, in both corn and prairie systems, aggregate profiles have been reported to change dramatically over the course of just one growing season with changes in precipitation and enzyme activity (Bach & Hofmockel, 2015). Our results therefore do not necessarily contradict prior studies that found that elevated CO_2 led to increased aggregate formation over longer timelines (Rillig *et al.*, 1999a; 2002), but instead may just reflect the dynamic changes in aggregation over short timelines.

However, why we observed an effect of elevated CO_2 on aggregation in the first year, but not the second, is a bit of a puzzle (especially so due to some data constraints). One possible explanation is that microbial growth in the bags was just greater in the first year due to more favorable conditions in the plots. Transient differences in soil moisture or temperature, as well as availability of C substrates, could have led to differences in microbial growth between the two years. Unfortunately, our first-year samples were

compromised so we could not directly test this. However, we used climate data from Cedar Creek and plot-level data from BioCON to see how overall growth conditions might have differed between years and by treatments. There was about 25% more cumulative rainfall in the first year's growing season (April-October, when bags were harvested) compared to the second (583.4 mm and 434.1 mm, respectively) and daily highs during the growing season were ≈2C warmer in the first year (Welch Two Sample t-test, P=0.0171). The wetter and slightly warmer first year could have been more conducive for microbial biomass growth and activity generally, which could have made it more likely that microbes colonized the bags, and led to treatment effects on aggregation. In contrast, plot-level soil conditions were not particularly different between the two years (Table S2), although there were some trends that seemed consistent with our aggregate responses, despite being only marginally significant or not significant. For example, in the first year, there was a trend towards higher soil moisture under elevated CO_2, compared to ambient CO_2, but there was no difference with CO_2 in the second year (ANOVA CO_2*Year P=0.0806, Fig. S8). In addition, although not statistically significant (P>0.1), annual root production trended slightly higher in elevated CO_2 plots in the first year of our experiment, but showed no difference with CO_2 in the second year (Table S2 and Fig. S9).

Another hypothesis for the difference in aggregation response between years is that microbial biomass in the bags could have been limited in the second year, and therefore less able to influence aggregation, due to depletion of labile C substrates in the bags.

Given the nature of the in-growth bags, they were effectively restricted from receiving new inputs of C, except for diffusion of soluble C into the bags, which was likely limited since C would have had to diffuse horizontally into the bags. As the microbial community colonized the bags and increased its biomass during the first year, it might have depleted the available C. Although microbial colonization into bags likely reflected the nearby source population, which would have been sensitive to CO_2 and N effects on soil moisture and C availability, biomass differences in the bags may only have been possible to detect with enough labile C present to support the microbes that did colonize. In the second year, there may have again been colonization that reflected plot-level differences in microbial biomass, however given the reduced C availability in the bags, the microbial community may have just been too C-limited to grow. If that were the case, that would also mean less production of fungal hyphae or bacterial products, like EPS, capable of facilitating aggregation in the second year. Again, unfortunately, due to the loss of the first year's samples, we were unable to compare microbial biomass from the second year to the first year to test this idea. The PLFA and NLFA data from the second year, however, revealed very low microbial biomass consistent with this idea. Although we cannot say for certain, it is possible that microbial biomass in bags was higher in the first year and more reflective of plot-level differences with CO_2 and N treatments.

In-growth bag treatment

Generally, the microbial biomass data from the second year suggest the microbial manipulation was not effective at excluding fungi in the smaller-mesh bags, as was

intended, at least in the second year (Table 3A). It is possible that saprotrophic fungi were able to colonize into the small mesh bags: although 1μm is too small to allow arbuscular mycorrhizal spores to enter, some saprotrophic fungi have hyphae as small as 1μm in diameter (Peay *et al.*, 2008). Perhaps the entry of fungi in the smaller-mesh bags intended to exclude them would have had a relatively small impact on the overall intended community in years with conducive growth conditions or when bags contain enough labile C to support microbial growth (as described above).

Despite the artificial nature of the in-growth bag environment, we did find changes in soil aggregation in the first year. These increases in aggregation occurred despite roots being absent, which furthers the idea that microbes can have a significant influence on mechanisms of physical C protection (Oades, 1993; Jastrow *et al.*, 1998; Rillig & Mummey, 2006), even if we cannot say definitively whether this was due to fungi or fungi and bacteria. Relatedly, although aggregation can increase with more fine root biomass, roots mainly increase aggregation via their effects on mycorrhizal abundance (Miller & Jastrow, 1990).

The in-growth bags used here also excluded soil animals, which could influence aggregation directly or through their influence on the microbial community. Earthworms, for example, have been shown to increase aggregation, as their processing of soil can lead to particle binding and C protection (Bossuyt *et al.*, 2005). Alternatively, the strictly microbe-mediated effects on aggregation observed here could be altered by inclusion of

the whole soil food web. In studies focused on decomposition, soil animals have been found to be important in determining microbial decomposition of organic matter (Crowther *et al.*, 2015). Compared to our experimental bags, inclusion of other soil animals may dampen microbial impacts on aggregation, as predation of microbes could lower their overall biomass in soils. For example, Allen *et al.* (2005) observed increased fungal grazing by mites at elevated CO_2 in a chaparral system (Allen *et al.*, 2005). Alternatively, depending on which soil animals are present and predominant, their inclusion could facilitate more aggregation (e.g. earthworms' positive effects on aggregation). However, the work we present here offers insight into how sensitive microbial effects on aggregation may be under global change, particularly in a sandy system where biotic influences on aggregation may be particularly important.

Conclusion

In low-clay soil systems, biotic controls on aggregation are likely especially important, and when changes in microbial communities or activity occur, as with environmental change, there could be related responses in soil structure. We used a novel approach intended to separate soil bacteria and fungi *in situ* to test how they uniquely influence aggregation and protection of C under elevated CO_2 and N addition. We found that after one growing season, microbially mediated aggregation increased under elevated CO_2. Although higher soil moisture under elevated CO_2 contributed to increased aggregation, the CO_2 effect may be due to increased C input belowground and associated increases in microbial growth and activity. We also found greater aggregation in the first year in

larger-mesh in-growth bags, intended to include fungi, compared to bags where fungi were physically excluded. However, after the second growing season these treatment effects on aggregation were no longer present, and both smaller-mesh and larger-mesh bags had been equally colonized by fungi. These findings highlight both the potential for change in soil structure and increased protection of C in response to elevated CO_2, but also the fact that aggregation is dynamic.

Acknowlegments

This work was supported by funding from the Cedar Creek Long Term Ecological Research Program (NSF DEB-9411972, DEB-0080302, DEB-0620652, and DEB-1234162), and CEK received support from a NSF Graduate Research Fellowship (00039202), UMN Pletcher Fellowship, UMN Carolyn Crosby Fellowship, and UMN Florence Rothman Fellowship. Thanks to Kally Worm for BioCON experiment support, Maria Rebolleda-Gomez for assistance with field work, Elizabeth Bach and Charlotte Riggs for insightful discussions on soil aggregation, and members of the Hobbie Lab and Forest Ecology Lab Group at the University of Minnesota for helpful feedback.

Table 1.1. ANOVA table for aggregate mean-weighted diameter (MWD) and aggregate fractions, as proportions of total soil mass. GWC is gravimetric water content at the time soils were sieved. 2013 aggregate MWD was log-transformed and aggregate fractions were logit-transformed across both years to meet model assumptions.

Effect	***MWD***		**Large macroaggregates (>2mm)**		**Medium macroaggregates (1-2mm)**		**Small macroaggregates (1mm - 250µm)**		**Microaggregates (>250µm)**	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
CO_2	*				**		*		**	
Nitrogen				*						*
Bag	***		†		**	*	**		**	†
GWC	***	***	***	***	***	***	***	***	***	***
CO_2 * Nitrogen				*						
CO_2 * Bag		†				*				*
CO2 * GWC					*	***			***	*
Nitrogen * Bag										
Nitrogen * GWC					*	**				†
Bag * GWC			*							
CO_2 * Nitrogen * Bag										
CO_2 * Nitrogen * GWC	*				†				*	
CO_2 * Bag * GWC						†	†			*
Nitrogen * Bag * GWC							†			
Marginal R^2	0.65	0.82	0.43	0.71	0.68	0.72	0.45	0.23	0.77	0.82
Conditional R^2	0.72	0.86	0.48	0.72	0.70	0.75	0.53	0.36	0.79	0.84

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 1.2. ANOVA table for aggregate fraction C content, as proportions of total C, across time. Proportion of total C in each aggregate fraction was logit-transformed to meet model assumptions.

Effect	C in Large macroaggregates (>2mm)		C in Medium macroaggregates (1-2mm)		C in Small macroaggregates (1mm - 250µm)		C in Microaggregates (>250µm)	
	2012	2013	2012	2013	2012	2013	2012	2013
CO_2			*		*			
Nitrogen		*		†		*		
Bag			*	**		*	*	†
GWC	***	***	***	***	***	***	***	***
CO_2 * Nitrogen		*						
CO_2 * Bag	†			*		†		
CO2 * GWC		†						**
Nitrogen * Bag								
Nitrogen * GWC	*				**			*
Bag * GWC			*					*
CO_2 * Nitrogen * Bag								
CO_2 * Nitrogen * GWC		†						**
CO_2 * Bag * GWC				**				**
Nitrogen * Bag * GWC				*				†
Marginal R^2	0.36	0.56	0.60	0.55	0.44	0.32	0.49	0.68
Conditional R^2	0.36	0.56	0.63	0.56	0.47	0.39	0.54	0.71

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 1.3. ANOVA table for treatment effects on total microbial biomass, and abundance (**A**) and relative abundance (**B**) of fungal and bacterial biomarkers from PLFA and NLFA. All biomarkers are from PLFA unless noted as from NLFA. Abundance of biomarkers (nmol/g soil) was log- or square root- transformed to meet model assumptions when needed. The group the biomarker indicates is in parentheses: arbuscular mycorrhizal fungi (AMF), general saprotrophic fungi (GF), Gram positive bacteria (Gram +), and Gram negative bacteria (Gram-).

A.	**Total microbial biomass** [a]	**Lipid Abundance**							
Effect		**16:1ω5c (AMF)** [b]	**16:1ω 5c NLFA (AMF)** [b]	**18:1ω9c (AMF/GF)** [b]	**18:1ω9c NLFA (AMF/GF)** [b]	**18:2ω6,9c (GF)**	**18:2ω6,9c NLFA (GF)** [b]	**15:0 iso (Gram +)** [b]	**16:1ω7c (Gram -)** [b]
CO_2									
Nitrogen									
Bag						†			
CO_2 * Nitrogen									
CO_2 * Bag			†						
Nitrogen * Bag	*	†		*				*	*
CO_2 * Nitrogen * Bag									
Marginal R^2	0.23	0.24	0.17	0.21	0.06	0.21	0.03	0.23	0.22
Conditional R^2	0.23	0.24	0.17	0.21	0.06	0.21	0.03	0.23	0.26

B.	**Phospholipid Relative Abundance**				
Effect	**16:1ω5c (AMF)** [b]	**18:1ω9c (AMF/GF)** [b]	**18:2ω6,9c (GF)** [b]	**15:0 iso (Gram +)** [a]	**16:1ω7c (Gram -)** [b]
CO_2					
Nitrogen					
Bag				*	*
CO_2 * Nitrogen					
CO_2 * Bag		†	*		
Nitrogen * Bag	†			†	†
CO_2 * Nitrogen * Bag					
Marginal R^2	0.14	0.18	0.22	0.24	0.26
Conditional R^2	0.20	0.18	0.22	0.47	0.38

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; [a] log-transformed; [b] square root-transformed

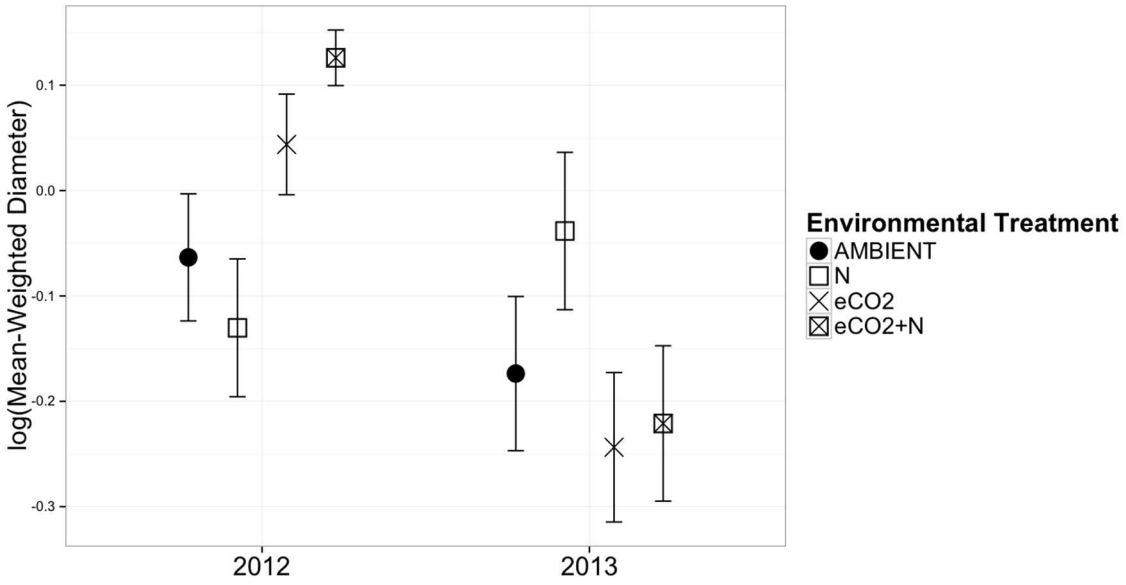


Figure 1.1. CO_2 and nitrogen effects on total aggregation, as mean-weighted diameter (MWD), over time. MWD is log-transformed to meet model assumptions.

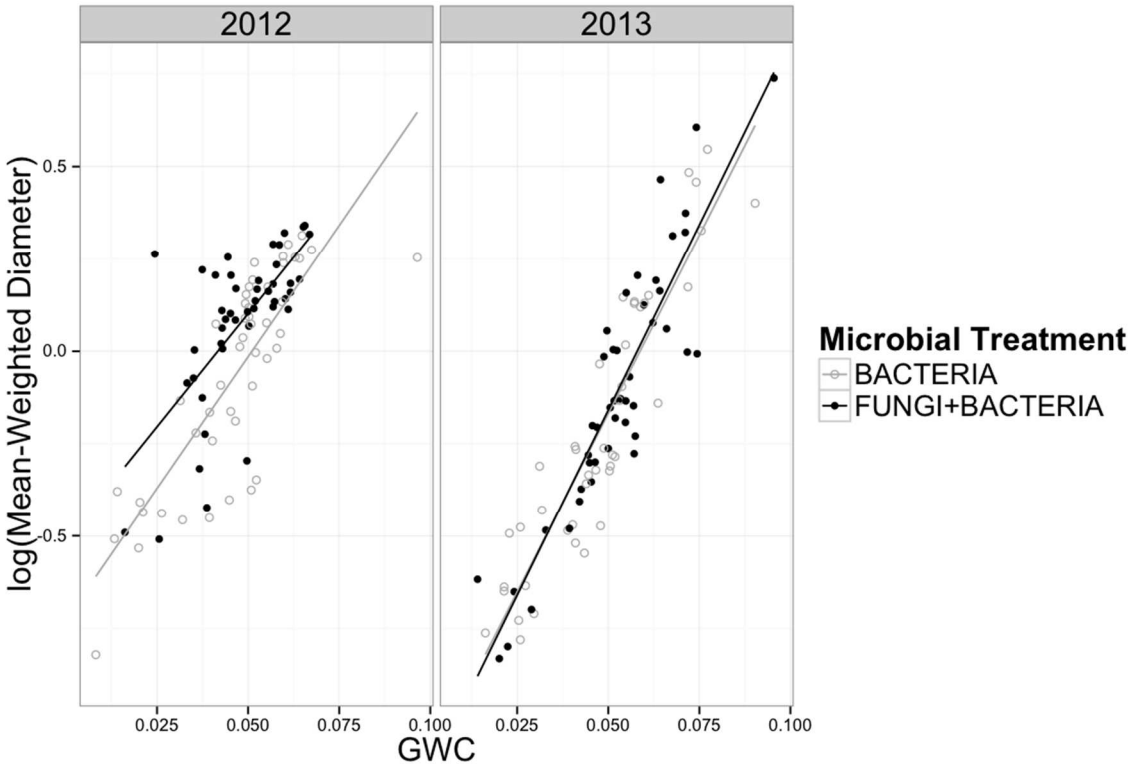


Figure 1.2. Aggregate mean-weighted diameter (MWD) across in-growth bag types and time, given soil gravimetric water content (GWC) at time of sieving.

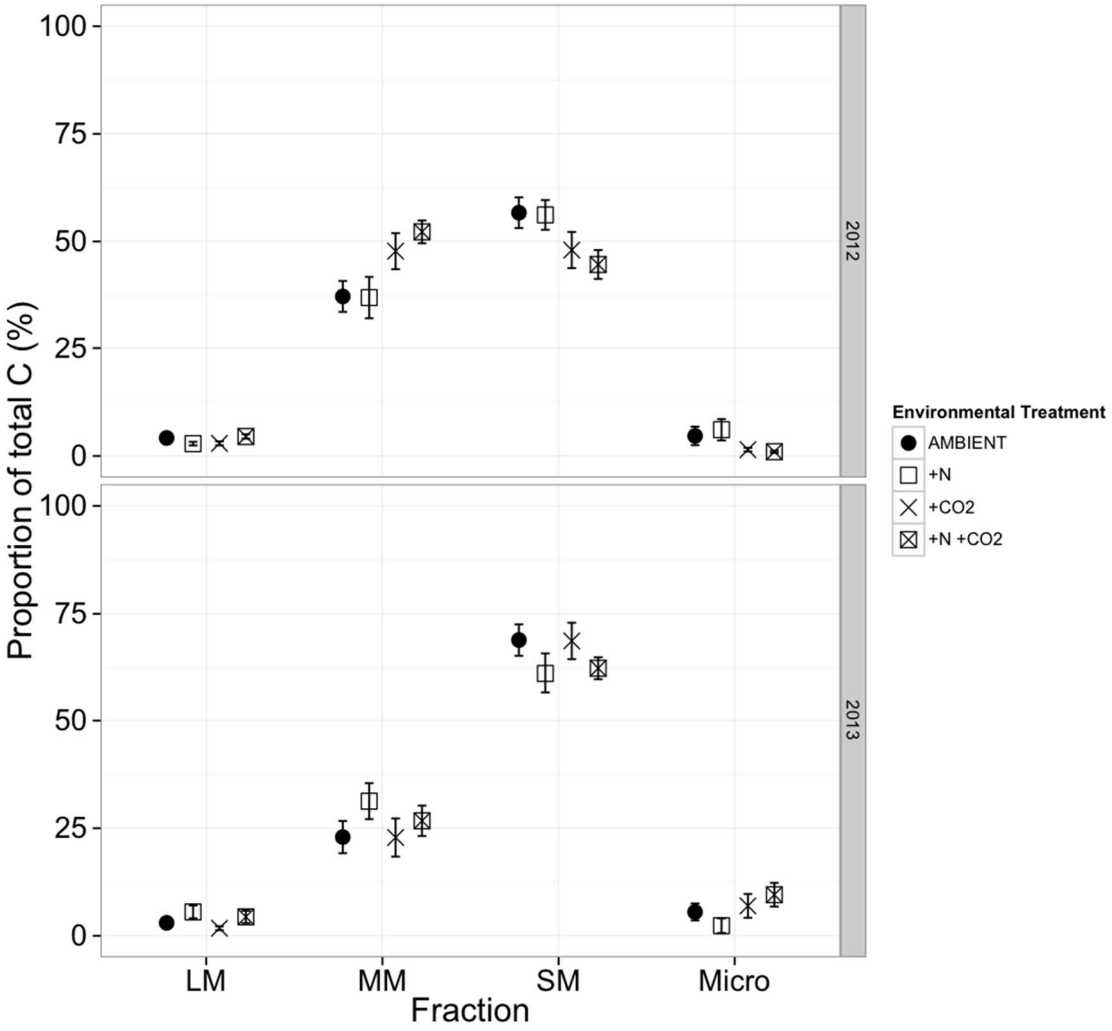


Figure 1.3. CO_2 and nitrogen effects on the distribution of C across aggregate fractions, over time.

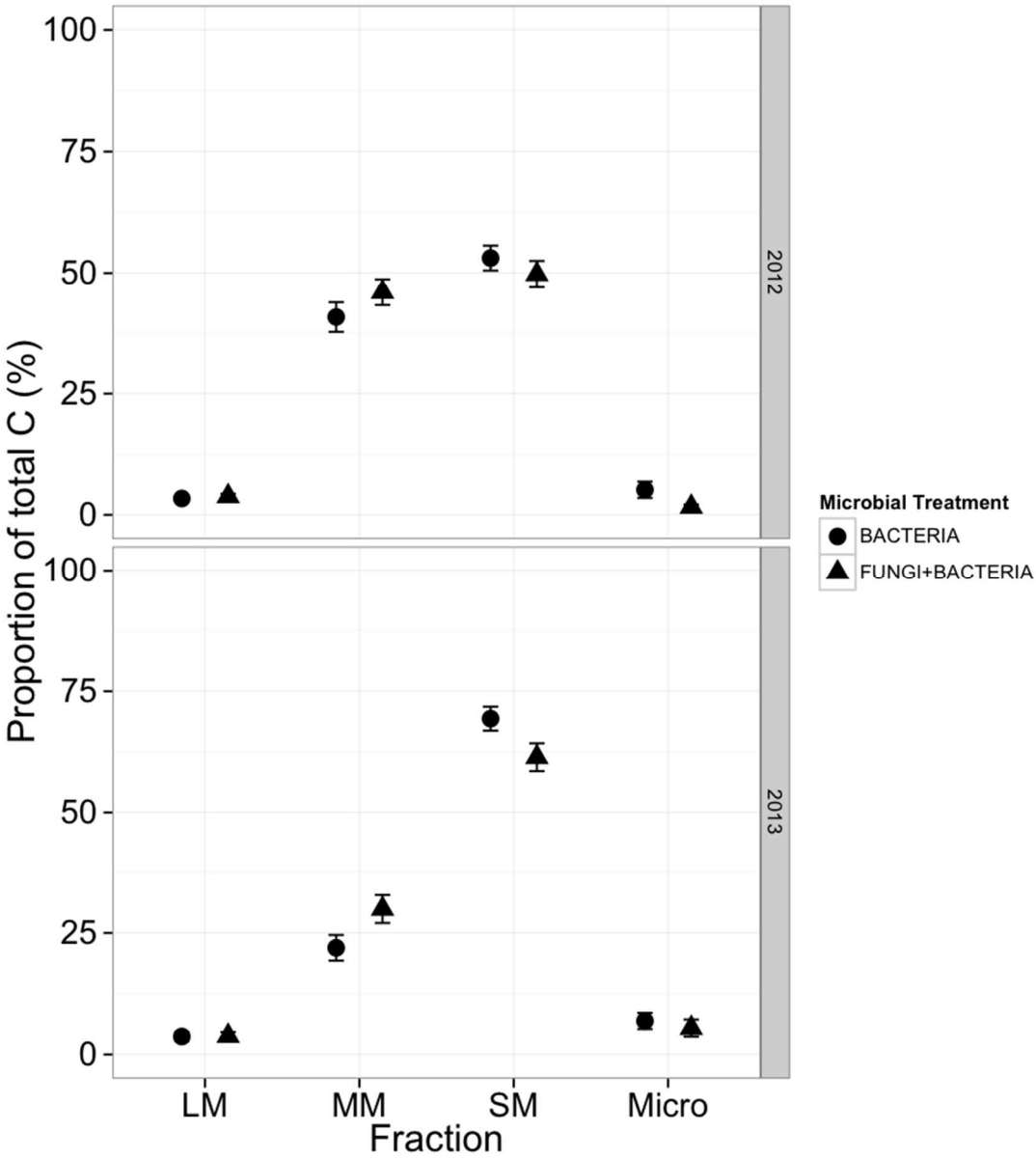


Figure 1.4. In-growth bag effects on distribution of C across aggregate fractions, over time.

CHAPTER 2

Long-term nitrogen addition does not increase soil carbon across different ecosystems in sandy soils

Abstract

By 2050, human activity will have almost doubled terrestrial inputs of reactive nitrogen (N), compared to pre-Industrial Revolution levels. It has become commonly accepted that, in addition to its positive effects on primary production, increased N deposition could promote carbon (C) accumulation in soils by reducing microbial decomposition of soil organic matter. However, this conclusion is based largely on studies from temperate forest systems and whether N deposition has similar effects on soil C across ecosystems with different dominant vegetation remains untested due to confounded differences in climate and soil type in prior empirical studies. Our goal was therefore to test the effects of sustained N additions on soil C pool sizes and cycling across different ecosystem types while controlling for soil type and climate. We used a 12-year N fertilization experiment at the Cedar Creek Ecosystem Science Reserve in central Minnesota, USA that included grassland, conifer and deciduous forest sites. We conducted a year-long microbial respiration incubation with soil from these sites, fit one- and two-pool decay models to respiration data to identify C pool sizes and decay rates, and assessed effects of N addition, site, and soil characteristics on C cycling. In contrast to previous studies, N addition consistently had no effect on soil %C, cumulative microbial respiration, soil C

pool sizes, or rates of decay across ecosystem types. The lack of response in soil C cycling occurred despite N-induced declines in soil pH, as well as site-related changes in microbial biomass, and decreases in fine root C:N and lignin:N. These results suggest that the effect of N on soil C pool sizes and cycling are likely highly location- and ecosystem- specific, raising caution for broad extrapolation of results from individual systems to global models.

Introduction

Soils represent the largest global terrestrial pool of carbon (C) (Ciais *et al.,* 2013) such that even small shifts in soil C pools could have large implications for atmospheric concentrations of carbon dioxide (CO_2). Increasing availability of nitrogen (N) in ecosystems has implications for soil C pools, as N is intricately connected to primary production (Vitousek & Howarth, 1991; LeBauer & Treseder, 2008), soil microbial biomass (Treseder, 2008), and decomposition (Berg, 2014). With global anthropogenic inputs of biologically reactive N up twelve-fold since 1860 (due to agricultural practices, fertilizer use, and fossil fuel combustion), and expected to continue to rise (Gruber & Galloway, 2008), N addition could lead to measurable changes in soil C. However, since ecosystem type is often confounded with soil texture or climate, whether soil C cycling across different ecosystem types (e.g. deciduous forests, coniferous forests, and grasslands) responds similarly to N addition remains poorly understood. Here we focus on teasing out the effects of N enrichment on soil C dynamics across a range of ecosystems with the same soil and climate.

The magnitude, and direction, of soil C responses to N addition differ substantially across locations and ecosystem types. The majority of studies assessing N effects on soil C cycling come from temperate forest systems (Jian *et al.,* 2016), and mainly report lower microbial respiration (Janssens *et al.,* 2010) and greater soil C accumulation with added N (Pregitzer *et al.,* 2007; Jian *et al.,* 2016). Although less common, grassland experiments testing effects of N addition also have reported reductions in microbial

respiration (Riggs *et al.,* 2015) and increases in total soil C (Fornara & Tilman, 2012; Cenini *et al.,* 2015), but also neutral effects on both microbial respiration (Riggs *et al.,* 2015) and total soil C (Zeglin *et al.,* 2007). Yet, the variation in C responses to N addition can be quite large: for example, although a meta-analysis of 36 studies from temperate forests found an average 15% reduction in microbial respiration with N addition, the responses ranged dramatically from a 57% suppression to a 63% increase (Janssens *et al.,* 2010). Another meta-analysis found that soil C responses to N addition were more positive under higher cumulative N load, and higher N application rates (Lu *et al.,* 2011). However, among these studies, dominant plant species also differ substantially and it is not well understood how generalizable effects of N enrichment are by specific ecosystem type. Indeed, studies comparing soil carbon responses to N addition in multiple forests with different dominant canopy species have found consistent positive effects of N (Frey *et al.,* 2014), as well as both positive and negative effects of N addition (Waldrop *et al.,* 2004). Here ecosystem properties, such as litter chemistry, soil pH, and microbial biomass, could lead to distinct N-addition effects not only on microbial respiration and total soil C, but C pools with different mean residence times as well.

Soil C pools with different mean residence times should respond differently to N addition (Neff *et al.,* 2002; Reid *et al.,* 2012; Riggs *et al.,* 2015) since their rates of cycling have unique controls, which could be influenced differently by N and ecosystem type. Specifically, decomposition of the fast-cycling, labile C pool (C_f), that is physically and chemically accessible to microbes, is controlled mainly by environmental conditions

(Trumbore, 1997), litter chemistry (Cleveland *et al.,* 2014), and microbial physiology (Schimel & Schaeffer, 2012). And although N addition is unlikely to significantly alter local temperature and moisture conditions, it can increase root N concentrations (Knops *et al.,* 2007) and alleviate microbial nutrient limitation, resulting in increased microbial efficiency (Ågren *et al.,* 2001; Schimel & Weintraub, 2003; Manzoni *et al.,* 2012). Indeed decomposition of fast-cycling C has been shown to increase with N addition (Neff *et al.,* 2002; Riggs *et al.,* 2015). In contrast, slow-cycling C (C_s) is controlled by physical and chemical protection (Jastrow *et al.,* 2006; Dungait *et al.,* 2012; Angst *et al.,* 2017) and has been shown to decay more slowly with added N (Riggs *et al.,* 2015). N addition could decrease the decay rate of slow-cycling C (k_s) by facilitating soil aggregation (Riggs *et al.,* 2015), reducing C mineralization within aggregate fractions (Tan *et al.,* 2017), or affecting the capacity for organic matter stabilization via cation bridging. Specifically N-induced acidification (Bouwman *et al.,* 2002) could lead to leaching losses of base cations (Aber *et al.,* 1998), but also increased solubility of polyvalent cations like Al^{3+} and Fe^{3+}, which strongly bind negatively-charged organic matter to negatively-charged soil mineral surfaces protecting it from decomposition (Hobbie *et al.,* 2007). However since ambient pH levels differ substantially by vegetation cover (Reich *et al.,* 2005; Mueller *et al.,* 2012), and sites with low cation exchange capacity can be more sensitive to N addition (Clark *et al.,* 2007), whether added N induces strong acidification – and thus increased organic matter stabilization – may depend on the ecosystem type. Combined, total microbial respiration has consistently been shown to decrease with N addition, which has been linked to N's negative impact on total microbial biomass (Riggs

& Hobbie, 2016). N addition can decrease microbial biomass (Treseder, 2008), yet since microbial communities differ across ecosystems with different dominant plant species and fertility (Wardle, 2004), the magnitude of microbial responses to N addition could depend on the ecosystem (Leff *et al.,* 2015).

Therefore, our objective with this study was to assess how N addition affects soil C cycling across different ecosystem types. Despite a breadth of studies testing soil C responses to N addition, other comparative experiments that control for soil type, climate, and N deposition history are, to our knowledge, non-existent, but important for determining whether N addition influences soil C cycling similarly across different ecosystem types. The diverse ecosystems within the 22 km^2 of the Cedar Creek Ecosystem Science Reserve in central Minnesota present a unique opportunity to test this, as plant species composition varies while soil type, climate, and N deposition history are held constant. Additionally, the small experimental plots we used allowed us to focus on N enrichment effects on decomposition processes while likely minimizing N-induced changes in C inputs from productivity responses. We hypothesized that N addition would i) increase total soil C concentration by ii) reducing microbial respiration, and specifically, that iii) decay rate of the smaller fast pool of C (k_f) would increase, from alleviating microbial nutrient limitation, and iv) decay rate of the larger slow pool of C (k_s) would decrease, from greater potential for cation bridging due to N-induced soil acidification and enhanced solubility of polyvalent cations such as Al^{3+} and Fe^{3+}. However, we expected sites to differ in the magnitude of their responses due to plant

cover-induced differences in ambient root chemistry, microbial biomass, and soil pH. Specifically, with N addition we expected sites with high root C:N and lignin:N to have greater increases in fast pool decay rate (k_f) because their microbial communities may be particularly nutrient limited, and sites with low ambient pH to show less of a response in slow pool decay (k_s) because they could have already experienced some acidity-induced weathering.

Materials and Methods

Study Site

Experimental plots were established in 1999 across eight sites of differing vegetation cover at the Cedar Creek Ecosystem Science Reserve in Bethel, MN (latitude 45.40°N, longitude 93.20°W, elevation 270m) (Hobbie, 2005). From 1999-2011, average annual precipitation was 744 mm/yr and mean annual temperature was 7.2 °C. The eight specific sites, all within 5 km of each other, included different canopy assemblages: 2 pin oak stands (*Quercus ellipsoidalis*), 2 white pine stands (*Pinus strobus*) (one plantation, Pine 1, and one natural stand, Pine 2), 1 maple-basswood stand (*Acer saccharum*, *Tilia Americana*, and *Quercus ellipsoidalis*), 1 clonal bigtooth aspen stand (*Populus grandidentata*) that had invaded an old field, and 2 abandoned agricultural fields now dominated by tallgrass prairie species (mix of C3 and C4; Old Fields 1 and 2) (Hobbie, 2005, 2008). The sites are all on a sandy outwash plain (>90% sand), and soils are designated as Udipsamments (Grigal & Homann, 1994). At each site 12 2.5m by 2.5m plots were set up and randomly assigned to either the N-fertilized treatment (receiving a

total of 10g N/m^2/year as NH_4NO_3, applied three times over the growing season) or control (receiving equal amounts of water instead). Given the size of the plots, changes in vegetation composition only occurred in the grassland sites (N-enriched plots shifted from being dominated by *Schizachyrium scoparium* and other C3 and C4 grasses to being dominated by quack grass (*Elymus repens*). In all sites we determined whether N addition led to differences in fine root %C, %N, and C:N as well as fine root C chemistry (see *Fine root chemistry* below for more detail).

Soil Sampling

Soil cores were taken in October 2011 after twelve years of treatment. Prior to taking soil cores, the organic layer was removed if present. Five cores (2cm diameter) were randomly collected per plot to 10cm depth, combined and homogenized. Soils were transported to the lab on ice and stored in the refrigerator for no more than 48 hours.

Site Characterization

Given their role in C cycling we quantified soil moisture, soil pH, bulk soil C and N content and C:N ratio, fine root biomass, and microbial biomass C and N and C:N ratio. Soils were passed through a 2mm sieve, during which time fine roots were picked out, saved, and frozen for fine root biomass analysis. Fresh soils were used to measure gravimetric soil moisture and microbial biomass C and N. Remaining soil was air-dried for at least 48 hours before further analyses. Gravimetric soil moisture, reported as percent of total dry soil, was determined by drying 10g of pre-weighed fresh soil at

105°C for at least 48 hours. Microbial biomass was assessed using chloroform fumigation (Brookes *et al.,* 1985). Two aliquots of fresh soil (equivalent to 10g dried soil) from each sample were extracted with 0.5M K_2SO_4 immediately or after 72 hours of chloroform fumigation in the dark. Extracts were immediately frozen and later measured for TOC/TN (Shimadzu TOC-V, Shimadzu Corporation, Kyoto, Japan). Microbial biomass C and N were determined by subtracting the non-fumigated sample from the fumigated sample. Results of chloroform fumigation are presented as chloroform-labile C and N, uncorrected for extraction efficiency. Soil pH was measured using a 2:1 water-to-soil method: we combined 10g air-dried soil and 20ml DI water, shook the sample for 30 minutes and let it sit for 1 hour, and then measured pH (ThermoScientific Orion 420A pH meter, Waltham, MA, USA; Hendershot *et al.,* 1993). Total soil C and N were measured via dry combustion of air-dried soils (Costech ECS 4010 Elemental Analyzer, Valencia, CA, USA). To determine fine root biomass, frozen roots were later thawed, washed with DI water, dried at 60°C for at least 48 hours, and then weighed.

Fine root chemistry

Fine roots were analyzed for C and N concentration and C chemistry. Dried roots were ground on a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) using a 0.85mm catch screen (standard size 20) and analyzed for C chemistry using an Ankom 200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) (% soluble cell contents, % hemicellulose and bound proteins, % cellulose, and % acid unhydrolyzable residue, lignin hereafter). Roots were further ground with a mortar and pestle and tested

for %C and %N via combustion (Costech CN Elemental Analyzer, Costech Analytical Technologies Inc., Valencia, CA, USA) using Atropine as a standard. We analyzed two analytical replicates per sample and took their average.

Soil C Decomposition

Soil C decomposition was assessed by measuring microbial respiration in long-term laboratory incubations. Within 48 hours of soil collection, 50g of fresh, root-free soil from each plot (n=6 treatment and n=6 control, from each of the eight sites) was weighed into a plastic cup and placed in a large quart-sized glass mason jar. Jars were covered with gas-permeable, low-density polyethylene film to avoid contamination and desiccation, and were stored in a dark room. Soil moisture was maintained throughout the incubation at 75% field capacity with routine re-wetting with DI water. Respiration was measured after a 24-hour incubation period 16 times over 385 days (on days 1, 4, 7, 12, 19, 31, 38, 44, 54, 68, 84, 124, 171, 251, 341, and 384 after soil collection). Jars were flushed to release built-up CO_2, capped, and headspace was then sampled using a syringe immediately and 24 hours after capping. The 24-hour CO_2 efflux was determined by the difference. Gas samples were analyzed using an infrared gas analyzer (LICOR LI-7000 CO_2 Analyzer, Lincoln, NE, USA). Cumulative respiration (mg C/g soil C and mg C/g soil) was determined using daily respiration at each sample point, accounting for days in between respiration sampling (i.e. by multiplying the average rates at t_1 and t_2 by the number of days between t_1 and t_2, following the methods of Riggs *et al.,* 2015).

Daily respiration rates were fit to both one-pool and two-pool decay models. For the one-pool model (Equation 1), C_t is the size of the entire C pool at time t and k is the rate of decay for the C pool. In contrast, in the two-pool model (Equation 2), C_f is the size of the fast pool and k_f is the decay rate of that fast pool. The second pool, the slow pool, which is the total C pool less the size of the fast pool (C_f), decays at rate k_s.

Equation 1 $$C_{rate}(t) = k*(C_t*e^{-kt})$$

Equation 2 $$C_{rate}(t) = k_f*(C_f*e^{-kft}) + k_s*((C_t-C_f)*e^{-kst})$$

Maximum likelihood estimation (MLE) was used to determine model parameters for C pools and decay rates at the plot (i.e. jar) level (bbmle package in R). One- and two-pool models were assessed using Akaike Information Criterion (AIC) values. Two-pool models were the better fit for 88.5% of samples (85/96), and one- and two-pool models were essentially indistinguishable (AIC < 2) for 8.3% of samples (8/96). Therefore, all results reported hereafter are from the two-pool models. Finally, because there are multiple parameters for each model, we tested the possibility of "parameter equifinality" where different combinations of parameters result in similarly good models (Beven, 2006). We found no evidence for equifinality (data not shown).

Statistical Analysis

Because we were interested in assessing the effects of N addition and site on various elements of C cycling, we developed linear statistical models to test their main and

interactive effects on cumulative respiration, and decay constants and pool sizes. Additionally, to account for as much response variable variation possible, we also developed statistical models for soil %C, microbial respiration, decay rates, and pool sizes that incorporated site characteristics along with N and site treatments as explanatory variables. For these models, we included site-level averages of ambient site characteristics, N treatment, site, a N treatment by site interaction, and each of the N treatment by average ambient site characteristic interactions. We only included site characteristics that were not highly correlated (r<0.8, see Table S1), which led us to include soil %C, soil C:N, microbial biomass C, and fine root biomass as explanatory variables in the models. Soil pH and %C were highly correlated (r = -0.85), which is why pH was left out. In all cases, data were checked to ensure model assumptions of normality and equal variance were met, and were natural log-transformed as needed to achieve model assumptions. All data analysis was done in R (version 3.0.2, The R Foundation for Statistical Computing).

Results

Differences in site characteristics across ecosystem types

Despite the relatively small and homogeneous geographic area of this study, we found substantial variation in ambient soil characteristics across sites, in some cases even under similar vegetation types (Tables 1 and 2). Soil N content in ambient plots ranged from 0.06% to 0.12%, with the grassland sites generally having lower soil N than the forested sites (with Pine 1 as an exception; Table 1). Soil C:N ratios were lower in the two

grassland sites (12.5 in Old Field 1 and 12.7 in Old Field 2) than in all the forested sites (which ranged from 16.5 in Oak 1 to 19.8 in Maple). Across the eight sites, ambient plots' microbial biomass C varied more than two fold and microbial N varied almost four fold (Table 1). The microbial biomass C:N ratio in ambient plots ranged from 5.0 to 6.2. Average site-level ambient soil moisture ranged from 4.4% to 9.3% (ANOVA Site $P<0.0001$ Table 1) and soil pH ranged from 5.1 to 5.7. Fine root biomass also ranged substantially across all sites (more than four fold), but tended to be similar within vegetation cover type, with lower fine root biomass in the oak- and pine-dominated sites. We used principle component analysis to visualize the site characteristics together, and found the two grassland sites clustered and the forested sites also mostly clustered together, although the pine sites, particularly Pine 1, stand out as being different from the other forested sites (Fig. 1). Despite this composite grouping, however, for individual site characteristics we found substantial variation even within the forested sites (Table 1).

Effects of N addition on soil characteristics across ecosystem types

Soil pH and N concentration were the only soil characteristics that differed consistently with N addition. As expected, soil pH was lower in plots with added N, but the magnitude of the effect differed by site with the two grassland sites showing larger effects (ANOVA, Treatment $P<0.0001$, Site*Treatment $P=0.0199$, Tables 1 and 2). Soil N concentration tended to increase in plots with added N (ANOVA, Treatment $P=0.0428$; Tables 1 and S2). At most sites, soil moisture was not affected by N addition, but the two grassland sites and one of the oak sites (Oak 1) did have lower soil moisture with added

N (ANOVA, Site*Treatment P=0.0465; Tables 1 and 2). N addition also influenced microbial C and N in some cases, but the effect depended on site (ANOVA Site*Treatment P=0.0002 and P = 0.0005, respectively; Tables 1 and 2): the two grassland sites and one of the oak stands (Oak 1) had lower microbial C and N with N addition, while the Aspen and Maple sites had higher microbial C with N addition (Table 1). The remaining soil characteristics – soil C:N ratio, fine root biomass, and microbial C:N ratio – were not affected by N addition (P>0.1).

Soil C concentration and cumulative microbial respiration

We found no effect of added N on soil %C (P>0.1), and although soil %C differed substantially by site (P<0.001) there was no interaction of added N x site for soil %C (Table 2). The average ambient soil C content ranged from 0.7% in Old Field 2 to 2.1% in the Pine 2 site. Overall, soil C tended to be lower in the two grassland sites than the six forested sites. However, there was some variation within ecosystem types: for example, the Pine 1 site, a plantation, had almost half the soil C content as the other white-pine dominated site, Pine 2, which was <1.5 km away.

Nitrogen addition had no consistent effect on cumulative microbial respiration, despite its increasing soil %N and reducing soil pH. When expressed per gram soil, the N effect on cumulative respiration depended on site (ANOVA Site*N interaction P=0.0204, Fig. 2): in most cases, N did not alter cumulative respiration, however the Norris grassland site had lower cumulative respiration (per gram soil) with N addition and the Pine 1 site had

higher respiration with N addition. When analyzed per gram soil C, there was no effect of N addition on cumulative respiration (ANOVA, N Treatment $P = 0.6845$; N*Site interaction $P = 0.2047$, Fig. 2 and Table S2).

Cumulative soil respiration differed significantly by site – both when analyzed per gram soil and per gram soil C. Average site-level cumulative respiration ranged almost three-fold from 0.75 mg C/g soil in Old Field 2 to 2.12 mg C/g soil in the Maple site (ANOVA, Site $P<0.0001$, Fig. 2). And although forested sites had higher cumulative microbial respiration than grassland sites, there were still large differences within the forested sites among vegetation cover types. Overall, cumulative respiration was greater with higher ambient plot-averaged bulk soil C concentrations and fine root biomass ($P<0.0001$ for both) and slightly lower with greater microbial biomass C ($P=0.0154$, $R^2=0.6499$, Table S3). Despite inclusion of these parameters in the model, there was still a marginally significant effect of site identity on cumulative respiration ($P=0.0797$). When analyzed on a per gram soil C basis, site still had a large effect on cumulative respiration (ANOVA, Site $P<0.0001$, Fig. 2 and Table S2). Overall, there was a small negative effect of ambient plot-averaged microbial C and very slight positive effect of ambient-level fine root biomass ($P= 0.0217$, $P<0.0001$, respectively). Again, despite inclusion of these parameters in the model, there was still a significant effect of site identity on cumulative respiration per gram soil C ($P= 0.0104$), suggesting there were other site-related factors influencing respiration that were not represented in the model.

Soil C Decomposition

Overall, long-term N addition had no influence on rates of C cycling. Across all ecosystem types, both the rate of decay of the fast pool (k_f) and the rate of decay of the slow pool (k_s) were not significantly affected by long-term N addition (ANOVA, P= 0.9482 and P= 0.8648, respectively, Fig. 3 and Table S2).

However, rates of decay of both fast and slow C pools differed significantly by site (ANOVA, k_f Site P<0.0001; k_s Site P<0.0001). The fast pool decay rate (k_f) ranged more than two-fold (from an average of 0.02 day^{-1} for Old Field 2 to an average of 0.06 day^{-1} for the Maple site) and k_s values ranged from an average of 0.00016 day^{-1} to 0.00026 day^{-1} (Old Field 2 and the Aspen site, respectively) (Fig. 3 and Table S2). There was not a clear pattern in k_f based on ecosystem type – the k_f values of the two grassland sites, for example, were as different as a grassland site and forested site or two forested sites (Fig. 3). However k_s values were more similar by ecosystem type: the two pine sites and the two oak sites had relatively matching k_s values, however the k_s values from the two grassland sites were still quite different (Fig. 3). Much of the variation (which was considerable in some cases) in site-level C cycling could be explained by measured soil characteristics, most notably soil %C (which was highly correlated with soil %N and soil pH, r=0.95 and r=-0.85, respectively, Table S1): soil %C was positively related to k_f (ANOVA, %C P=0.002, Table S3) and k_s increased very slightly with increasing soil %C (P= 0.0039). The slow pool decay rate, k_s, increased most with fine root biomass (P<0.0001) and decreased marginally with microbial C (P= 0.0210; Table S3). For both

fast and slow decay rates, however, site still had a significant effect after accounting for the influence of measured soil characteristics (Table S3).

Carbon pool sizes and composition

As with the decay constants, the pool sizes for both the fast (C_f) and slow (C_s) C pools did not change under long-term N addition (ANOVA, P= 0.5888 and P= 0.1229, respectively, Fig. 3 and Table S2). There was a marginal Site*N treatment interaction for C_f (ANOVA, P= 0.0775), where most sites showed no effect of N addition, but Old Field 2 had a smaller fast pool and the Pine 1 site had a larger fast pool with N enrichment. There was no interaction effect for C_s (P= 0.5089, Table S3). The ratio of fast to slow pool size also did not change with N addition and there was no significant interaction (ANOVA, N treatment P=0.3382, Site*N treatment P= 0.4278, Fig. S1). However, as with the rates of decay, pool sizes differed substantially by site.

The size of the fast pool (C_f) ranged by about two-fold across sites (the smallest site-level fast pool was from the Pine 2 site at 0.16 mg C g soil^{-1} and the largest was at the Norris grassland at 0.31 mg C g soil^{-1}) (ANOVA, Site P=0.0020, Fig. 3 and Table S2). Although there was not as much variation in C_f as in the fast pool decay rates, again, we found that sites with similar ecosystem types did not necessarily have the most similar C_f values (Fig. 3). When pooling across sites, the fast pool was smaller at higher soil %C (P=0.0173, Table S3). The model including site characteristics, however, only accounted for 15% of the observed variation (Table S3).

The size of the slow pool (C_s) also differed substantially by site (ANOVA, Site $P<0.0001$, Fig. 3 and Table S2). The forested sites had two- to three- times larger slow pools than the grassland sites, although there was still substantial variation in C_s within the forested sites (Fig. 3). The two pine sites, for example, had very different site-level average slow pools (the Pine 2 site was 20.8 mg C g soil^{-1} and the Pine 1 site was 12.3 mg C g soil^{-1}). Just over 80% of the observed variation in slow pool size was attributed to soil %C, such that C_s was larger with greater soil %C ($P<0.0001$, Table S3).

Fine root chemistry responses to N addition

Nitrogen addition influenced root chemistry, but not consistently across ecosystem types. Overall, root %N increased with N addition and there was a significant N*site interaction (ANOVA, $P<0.0001$ for both, Tables 3 and S4): in the grassland sites, fine root %N increased on average by 58% to 79%, and two of the forested sites also tended to have higher root %N (Maple and Oak 2). Fine root %C did not respond to N addition and there was no N*site interaction (ANOVA, $P=0.5523$, $P=0.5359$, respectively, and Tables 3 and S4), so there was a concurrent reduction in the C:N ratio in +N plots across sites, but driven mostly by the two grassland old field sites (ANOVA, +N $P=0.0002$, N*Site $P=0.0240$, Tables 3 and S4). The proportion of soluble cell contents, the most labile C, increased with N addition across all sites resulting in relatively comparable proportions across sites, but again the increase was markedly higher in grassland sites where they went up by almost one third (ANOVA, N treatment $P=0.0116$, Tables 3 and S4, Fig. S2).

Other C compounds (hemicellulose and bound proteins, cellulose, and lignin) did not change with N addition (ANOVA, $P>0.1$ Tables 3 and S4, Fig. S2). The ratio of lignin to N in fine roots was generally lower with added N (ANOVA, $P<0.0001$, Table S4).

Ambient fine root chemistry differed across ecosystem types, but less drastically than we expected. There was a significant effect of Site on root %N, but it was driven primarily by the Aspen site, which had lower average %N than the other sites (ANOVA $P<0.0001$, Tables 3 and S4). Fine roots also differed in their ambient %C by site, ranging from 24.5% at the Aspen site to 38.3% at Oak1 (ANOVA $P=0.0011$, Tables 3 and S4). As a result, the C:N ratio of fine roots also differed across sites (ANOVA $P<0.0001$, Tables 3 and S4). Sites also differed in the proportion of roots in soluble cell contents, hemicellulose and bound proteins, and lignin (but not cellulose) (ANOVA, $P<0.0001$, $P<0.0001$, $P = 0.0293$, $P = 0.4520$, respectively, Tables 3 and S4, Fig. S2). However, fine root lignin: N was not statistically different between sites (ANOVA, P= 0.2152, Table S4). Fine roots in ambient grassland plots had slightly lower amounts of soluble cell contents than forested plots, generally (except for Aspen), and higher hemicellulose and bound proteins content. Forested sites generally had higher lignin content than the grassland sites, but were still relatively variable, even between sites of similar ecosystem types (i.e. the two oak sites and two pine sites were as different to each other as to other forested sites) (Fig. S2).

Discussion

Contrary to our predictions that long-term N addition would reduce respiration rates and result in greater soil C, we found no overall effect of 12 years of N addition on soil %C, cumulative microbial respiration, or fast and slow pool decay rates or pool sizes. Moreover, the only differences in microbial respiration with N addition were when it was expressed on a per gram soil basis and were inconsistent: one of the two grassland sites had less cumulative respiration with N addition, and one of the two pine-dominated sites had more cumulative respiration with N addition. The consistent lack of effect on C cycling was surprising and occurred despite significant N effects on soil pH, soil %N, and microbial C and N (Tables 1 and 2). Here we explore possible explanations that collectively raise caution for generalizing results of N effects on soil C cycling.

No effect of N addition on C cycling across ecosystem types

Neither soil %C or cumulative microbial respiration per gram soil C responded to long-term N addition. Although our findings did not support our hypotheses, they confirm a prior study in a grassland experiment at Cedar Creek that also found no effect of N addition on microbial respiration (Riggs *et al.,* 2015). This lack of response could be due to an inconsistent microbial biomass response. Unlike previous studies (Treseder, 2008; Lu *et al.,* 2011; Liu *et al.,* 2015), we did not see a consistent decrease in microbial biomass with N addition across ecosystems: the two grassland sites and one of the oak stands (Oak 1) had lower microbial C and N with N addition, but the Aspen and Maple sites had higher microbial C with N addition (Tables 1 and 2). Yet, a recent study of

microbial respiration across grasslands in the Great Plains experiencing N enrichment found that reductions in respiration were due to concomitant reductions in microbial biomass (Riggs & Hobbie, 2016), as has been shown with soil CO_2 flux (Treseder, 2008). Although microbial respiration did not differ with N addition, we did see that sites with lower microbial biomass C under N addition also tended to have reduced microbial respiration with N addition, with the exception of Old Field 1 (Fig. S3). One question, then, is why our sites did not consistently demonstrate lower microbial biomass with N addition, despite ubiquitous declines in soil pH (Tables 1 and 2), which can inhibit microbial abundance and alter community composition (Rousk *et al.,* 2010). The lack of a broad microbial biomass response could relate to the lack of response of fine root biomass to N addition (Table 2), but likely not the root chemistry response (as lignin:N ratio decreased and root %N increased, particularly in the grassland sites, with N addition).

Although a lack of response in soil %C or cumulative respiration could have masked important patterns in decay rates and pool sizes of fast- and slow-cycling C (Neff *et al.,* 2002; Reid *et al.,* 2012), we again saw no change with N addition. We expected N enrichment to increase decay rates of the fast-cycling C (k_f) overall, by alleviating microbial nutrient limitation. The lack of response in k_f values occurred despite changes in microbial biomass (microbial C was lower at the grassland sites and Oak 1, and higher at the Aspen and Maple sites). We further hypothesized that sites with roots with high C:N and lignin:N would have greater increases in k_f , since their microbial communities

could be more nutrient limited, but we did not see a large range in fine root C:N or ambient root C chemistry (Fig. S2), and lignin:N did not differ between sites.

In contrast, we expected to find lower decay rates of slow-cycling C (k_s) with N enrichment because of N-induced soil acidification, and resulting changes in cation bridging. Furthermore, we thought sites with lower ambient pH would show less of a response, as they could have already experienced weathering under low pH. We did see a consistent reduction in soil pH with N addition across sites, but no response in k_s. The decay rate of the slow pool was significantly related to soil %C, which was also highly negatively correlated with pH. However, due to the correlation, we were unable to decipher pH's specific connection to C cycling. The lack of response in k_s could also relate to the lack of response in k_f. Reduced decay of the slow pool could occur with increased decay of fast-cycling C, where the increased abundance of microbial products actually leads to more long-term stabilization of those products on soil mineral surfaces. Consistent with this emerging framework (Cotrufo *et al.,* 2013), a 19-year N addition experiment in a temperate grassland found an increase in total soil C driven mostly by greater stabilization of C in the heavy fraction from enhanced β-1,4-glucosidase enzyme production (Cenini *et al.,* 2015). The lack of change in the decay rate of the fast-cycling pool (as noted above) could therefore be one possible explanation for a lack of response in k_s. Despite a consistent lack of response to N addition across all the soil C cycling measures tested, there are characteristics of Cedar Creek, and this study (explored below), that differ from other sites and studies and could help explain the discrepancies.

Soil characteristics may contribute to the absence of an N effect

It is possible that the very sandy soils at Cedar Creek may be less likely to demonstrate changes in chemical protection of organic matter with N-induced acidification. Despite N reducing pH substantially in our study by 0.1 to 0.6 pH, corresponding to a one- to four-fold increase in acidity (Tables 1 and 2), the soil has very little clay content and is mostly sand (>90%; Grigal, 1974). Sandy soils, like those of Cedar Creek, have a low surface area and a low cation exchange capacity, and therefore a low potential for chemical stabilization of organic matter. Additionally, N-induced acidification may not have increased cation availability for organic matter bridging in a meaningful way given the soil's low clay content, which is the source of polyvalent cations Al^{3+} and Fe^{3+}. As a result, acidification effects on chemical protection of organic matter might have been negligible in this study. This could partially explain the lack of C_s and total microbial respiration response since N-induced soil acidification has been shown to be the primary control of microbial respiration, not N addition per se (Chen *et al.,* 2015). However, it is worth noting that other high-sand sites studied by Zak *et al.* have demonstrated a reduction in microbial respiration with N addition (2017). Although these sites had only ~5% less sand content than Cedar Creek soils (~85% compared to >90%), the slightly greater silt and clay content was enough to result in substantial increases in occluded particulate organic matter under N addition (Zak *et al.,* 2017).

The low nutrient content of Cedar Creek soils could also have contributed to the lack of a

heterotrophic respiration response. Across temperate forest sites, microbial respiration has been linked to site-level net primary production, where more productive sites tend to show higher respiration and a greater inhibitory effect of N addition (Janssens *et al.,* 2010), and some low-N sites have shown no effect of N addition on heterotrophic respiration (Kang *et al.,* 2016). Related, in a cross-site grassland study, the two out of five sites that demonstrated lower microbial respiration with N addition, also happened to have higher soil N content, although the specific role of site-level N was unclear as these sites also had lower % sand than Cedar Creek (substantially so in one of the sites) (Riggs *et al.,* 2015). One explanation for a reduced effect of N addition at low-nutrient sites is that there could be greater microbial nitrogen limitation, and therefore immobilization (Janssens *et al.,* 2010). However, if that were the case in our experiment we would expect to see total microbial biomass N to increase with N addition, which we did not see consistently (Tables 1 and 2): microbial N was higher in Pine 1, trended higher in the Aspen and Maple sites, and actually decreased with N addition in the two old fields and in Oak 1.

Another important distinction between soils studied here, and those considered in other studies, is their horizon of origin. Most Cedar Creek soils lack a significant organic horizon and in most cases, including the soils at sites used in this study, have no organic horizon (Grigal, 1974). In studies that test the effect of N on soil C cycling and differentiate by horizon, the positive effect of N addition is most common in the organic soil horizon, and not often observed in mineral soil (Liu & Greaver, 2010; Frey *et al.,*

2014; Maaroufi *et al.,* 2015), or the horizon is not reported (Janssens *et al.,* 2010). For example Frey *et al.* (2014) found positive effects of N addition on C stocks, mostly through reductions in soil organic matter decomposition, but only in organic horizons of northern hardwood forests. And, despite seeing a consistent positive effect in the organic horizon, there was only a positive effect of N addition on mineral horizon C at one of two studied sites (dominated by mature red pine), and only at the highest rate of N addition (150 kg N ha^{-1} yr^{-1}) (Frey *et al.,* 2014). Higher total C in the organic layer has been attributed to the positive impact of N addition on aboveground biomass production (Janssens *et al.,* 2010). In contrast, a lack of response in mineral soil could relate to there being less availability of microbial products that can be stabilized onto mineral surfaces (Cotrufo *et al.,* 2015). Although a recent study did demonstrate greater accumulation and stabilization of C in mineral soils under N addition, likely from enhanced microbial activity and availability of microbial products, the sites used had well-developed organic horizons (Zak *et al.,* 2017). It is possible that without such organic horizons at Cedar Creek there was generally less substrate available for which N-induced increases in microbial efficiency and release of products could occur, reducing the amount of C that could be stabilized in mineral soils (Cotrufo *et al.,* 2015). Since the positive effect of N on soil C does not appear consistently in mineral soil (Liu & Greaver ,2010), as confirmed in this study, and could be less pronounced in sandy soil, it is worth using caution in broadly extrapolating to include the effect in global C models (Zaehle & Friend, 2010) and in understanding future carbon-climate feedbacks (Heimann & Reichstein, 2008). Models that assume N addition increases *all* soil C could overestimate

C storage enhancement under increased N deposition, since much of soil C is at depth below the organic horizon (Jobbágy & Jackson, 2000).

Variation in soil C and cycling across ecosystem types

Despite controlling for climate, soil type, and N deposition history, we found substantial differences in soil %C, cumulative microbial respiration, decay rates, and pool sizes across ecosystem types. In some cases there was also as much difference between sites of a similar ecosystem type as across ecosystem types (particularly with the oak and pine stands). For example, Pine 1 appeared more like the grassland sites than the other pine site in various responses. This result was surprising and highlights how seemingly similar landscapes can behave quite differently. However, it is worth noting that land use history may have played an important role here. Several of the sites in this study were in cultivation previously – the two old-field grasslands, the Aspen site, and likely Pine 1 (it is a plantation and, given local history, was likely an abandoned old field before that). These sites likely have depleted soil C due to prior cultivation (McLauchlan *et al.,* 2006). In our study, soil %C explained much of the variation for most of the C cycling metrics studied – decay rates, and pool types (Table S3). This was expected since C decay metrics were analyzed per gram soil, however it may mask other important relationships since soil %C was highly positively correlated with soil %N, highly negatively correlated with pH, moderately positively correlated with soil C:N, and microbial biomass C and N (Table S1*).* Although pH has been linked to microbial communities (Rousk *et al.,* 2010) and microbial activity (Whittinghill & Hobbie, 2011), given the strong correlations with

%C, we cannot further discern the mechanisms behind the relationships with C cycling.

Conclusion

We consistently found no evidence for increased soil C accumulation with long-term N addition, performed on a small spatial scale such that it did not impact ANPP but did impact soil N availability and pH, across multiple ecosystem types where climate, soil type, and N deposition history were held constant. Our experimental method allowed us to minimize what, on larger scale, would be N impacts on ANPP, and thus explore how factors other than ANPP might influence soil carbon storage rates. Specifically, 12 years of N addition led to no change in soil %C, microbial respiration per gram soil C, fast- and slow-cycling C pools and decay rates. This ubiquitous lack of an effect occurred despite N-induced declines in pH across sites, and changes in microbial biomass C and N and decreased root lignin:N and C:N in some sites. The sandy, nutrient-pool nature of soils studied here could help explain the lack of response, since opportunities for organic matter stabilization on mineral surfaces are low and N-induced acidification may not substantially increase availability of polyvalent cations that create strong organic matter bridges. Additionally, our work confirms prior findings that reported no effect of N addition on soil C in mineral soils. Overall, the results reported here contradict the often-cited inhibitory effect of N addition on microbial respiration, and related build-up of soil C, and suggest that modeling efforts that assume that N addition leads to lower microbial respiration or greater soil C content across soil types and horizons could overestimate future C storage under increasing N deposition.

Acknowledgments

This work was supported by the Cedar Creek Long Term Ecological Research program (National Science Foundation (NSF) DEB-9411972, DEB-0080302, and DEB-0620652) and by an NSF CAREER award (DEB-0347103). CEK was personally supported by a University of Minnesota College of Biological Sciences Graduate Excellence Fellowship and a NSF Graduate Research Fellowship (00039202). Thanks to Chris Buyarski for assistance with experiment maintenance, as well as Peter Reich, Dave Tilman, Nancy Johnson, Jessica Gutknecht, Lauren Cline, Melissa Pastore, Craig See, Chris Walter, and Rachel King for helpful comments on manuscript drafts.

Table 2.1. Average soil pH, soil C, soil N, soil C:N ratio, soil moisture, fine root biomass, and microbial biomass C, N, and C:N ratio (MB C:N) in ambient (Amb.) and N addition (+N) plots across all sites. Soil C and N and soil moisture are in percent (%), fine root biomass is in g/m^2, microbial biomass C (MBC) and N (MBN) are in µg/g soil Means are reported with standard errors in parentheses (N=6, or *N=5).

	Old Field 1		Old Field 2		Aspen		Maple		Oak 1		Oak 2		Pine 1		Pine 2	
	Amb.	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**
pH	5.70	5.17	5.62	4.99	5.50	5.28	5.41	5.12	5.08	4.98	5.30	4.97	5.57	5.45	5.17	4.99
	(0.04)	(0.06)	(0.07)	(0.11)	(0.08)	(0.07)	(0.05)	(0.12)	(0.04)	(0.06)	(0.10)	(0.05)	(0.04)	(0.05)	(0.07)	(0.09)
Soil C	0.71	0.70	0.67	0.74	1.55	1.70	1.98	2.19	1.86	1.72	1.59	1.53	1.08	1.44	2.13	2.06
	(0.07)	(0.03)	(0.05)	(0.02)	(0.11)	(0.09)	(0.21)	(0.13)	(0.27)	(0.17)	(0.11)	(0.12)	(0.01)	(0.09)	(0.22)	(0.15)
Soil N	0.06	0.06	0.06	0.06	0.09	0.10	0.10	0.12	0.11	0.10	0.10	0.09	0.06	0.07	0.12	0.12
	(0.01)	(0.00)	(0.01)	(0.00)	(0.01)	(0.01)	(0.01)	(0.00)	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.01)	(0.01)	(0.01)
Soil C:N	12.52	11.60	12.72	13.23	17.26	16.42	19.84	18.85	16.49	16.55	16.57	16.14	19.04	19.88	17.83	16.74
	(0.22)	(0.29)	(0.95)	(0.34)	(0.32)	(0.29)	(1.03)	(0.89)	(0.51)	(0.50)	(0.34)	(0.42)	(0.36)	(0.45)	(0.25)	(0.28)
Soil moisture	6.30	5.24	5.91	4.58	8.46	8.52	9.17	9.63	9.31	7.52	7.21	7.53	4.43	4.87	7.17	7.93
	(0.42)	(0.43)	(0.36)	(0.27)	(0.31)	(0.29)	(0.39)	(0.30)	(0.68)	(0.44)	(0.42)	(0.58)	(0.10)	(0.32)	(0.77)	(0.80)
Fine root biomass	1,019.12	1,405.97	1,331.80	874.08	1,375.20	1,495.63	981.98	1,368.30	709.94	747.28	714.39	907.39	291.89	481.18	356.61	552.69
	(247.65)	(330.39)	(255.58)	(298.07)	(210.83)	(290.49)	(77.73)	(125.65)	(53.48)	(113.95)	(92.04)	(146.88)	(69.06)	(90.92)	(99.12)	(112.35)
MBC	97.15*	71.67*	117.41	57.79	155.69	188.45	194.18	251.19	175.87	134.28	158.45	168.56	84.31	107.79	127.86	129.70
	(10.26)	(4.51)	(8.56)	(9.04)	(10.86)	(10.58)	(8.39)	(22.94)	(12.46)	(19.15)	(3.95)	(17.23)	(8.60)	(8.26)	(17.55)	(14.80)
MBN	18.93*	11.88*	20.31	10.46	32.78	37.01	38.13	42.10	35.43	24.60	30.91	32.65	13.56	19.06	24.67	23.90
	(1.65)	(1.58)	(1.43)	(0.66)	(1.95)	(1.42)	(1.97)	(2.67)	(2.47)	(2.80)	(1.17)	(3.44)	(1.20)	(1.87)	(3.70)	(3.57)
MB C:N	5.12*	6.37*	5.78	5.35	4.74	5.09	5.11	5.92	4.98	5.41	5.14	5.18	6.21	5.74	5.32	5.57
	(0.21)	(0.54)	(0.12)	(0.69)	(0.09)	(0.24)	(0.14)	(0.19)	(0.17)	(0.17)	(0.13)	(0.10)	(0.20)	(0.24)	(0.35)	(0.21)

Table 2.2. ANOVA Table for soil pH, soil C, soil N, soil C:N ratio, soil moisture, root biomass, and microbial biomass C, N, and C:N ratio. Two-way ANOVAs were performed for each site characteristic and its response to Site and N Treatment (characteristic ~ Site*N).

Characteristic	*Site*	*Treatment*	*Site*Treatment*	*Adjusted R^2*
Soil pH	***	***	*	0.5428
Logged Soil %C	***			0.8007
Logged Soil %N	***	*		0.6792
Logged Soil C:N	***			0.7873
Soil moisture	***		*	0.6644
Fine root biomass	***			0.4458
Microbial biomass C	***		***	0.7001
Microbial biomass N	***		***	0.7382
Logged Microbial biomass C:N	†			0.1088

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 2.3. Fine root chemistry in ambient (Amb.) and N addition (+N) plots across all sites. Means are reported with standard errors in parentheses (N=6).

	Old Field 1		Old Field 2		Aspen		Maple		Oak 1		Oak 2		Pine 1		Pine 2	
	Amb.	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**	**Amb.**	**+N**
Fine root %C	26.56 (4.48)	24.93 (2.21)	31.46 (3.69)	36.16 (3.69)	24.48 (3.86)	23.03 (4.02)	28.63 (2.87)	33.26 (1.88)	38.26 (1.58)	30.47 (3.43)	27.21 (2.91)	31.52 (2.52)	34.61 (4.55)	38.95 (3.56)	34.98 (3.63)	35.98 (4.05)
Fine root %N	0.75 (0.09)	1.19 (0.09)	0.64 (0.05)	1.15 (0.07)	0.62 (0.03)	0.69 (0.09)	0.68 (0.07)	0.92 (0.05)	0.98 (0.02)	0.94 (0.07)	0.82 (0.05)	1.01 (0.07)	0.79 (0.08)	0.91 (0.04)	1.00 (0.06)	0.93 (0.05)
Fine root C:N	34.67 (3.59)	21.00 (1.16)	50.93 (8.64)	32.08 (3.14)	38.73 (5.23)	32.69 (2.61)	42.15 (1.81)	36.54 (1.90)	39.36 (2.19)	32.12 (1.93)	33.15 (2.71)	31.48 (2.71)	43.27 (4.01)	42.87 (4.17)	34.81 (1.80)	39.43 (4.79)
Fine root lignin:N	74.06 (12.13)	53.19 (9.31)	59.36 (7.77)	73.28 (22.69)	77.94 (8.36)	62.11 (4.17)	59.71 (4.27)	45.00 (6.05)	83.26 (22.34)	51.31 (6.27)	66.93 (13.26)	48.40 (6.11)	107.76 (21.29)	67.93 (10.58)	84.60 (13.36)	59.03 (12.83)
Fine root % soluble cell contents	15.64 (1.68)	21.64 (1.23)	15.27 (1.45)	19.99 (1.52)	15.69 (3.05)	20.08 (2.04)	19.34 (0.73)	19.47 (1.25)	24.30 (1.13)	21.14 (1.05)	18.85 (1.08)	20.08 (1.39)	26.42 (1.43)	26.70 (2.42)	19.57 (1.36)	22.74 (1.59)
Fine root % hemicellulose and bound proteins	16.27 (2.39)	13.75 (1.09)	16.50 (2.87)	18.18 (1.92)	9.73 (4.01)	7.62 (1.33)	7.63 (0.88)	6.68 (0.49)	8.32 (0.86)	6.22 (0.46)	6.06 (0.45)	6.93 (0.72)	6.24 (0.61)	7.68 (1.02)	5.98 (0.43)	7.34 (1.02)
Fine root % cellulose	19.38 (2.68)	14.47 (1.61)	18.63 (3.18)	21.43 (2.86)	15.43 (1.59)	15.04 (2.13)	18.85 (1.27)	18.32 (0.98)	19.25 (1.63)	16.14 (0.43)	15.86 (1.37)	17.35 (1.33)	17.24 (2.05)	19.39 (2.05)	14.53 (1.76)	20.19 (3.44)
Fine root % lignin	48.54 (6.44)	49.61 (3.76)	49.37 (7.16)	39.55 (6.09)	58.70 (3.98)	57.21 (4.53)	54.04 (2.08)	55.29 (2.34)	48.08 (2.20)	56.12 (1.62)	59.14 (2.38)	55.35 (2.84)	48.52 (3.95)	45.43 (5.47)	57.93 (4.55)	49.01 (5.97)

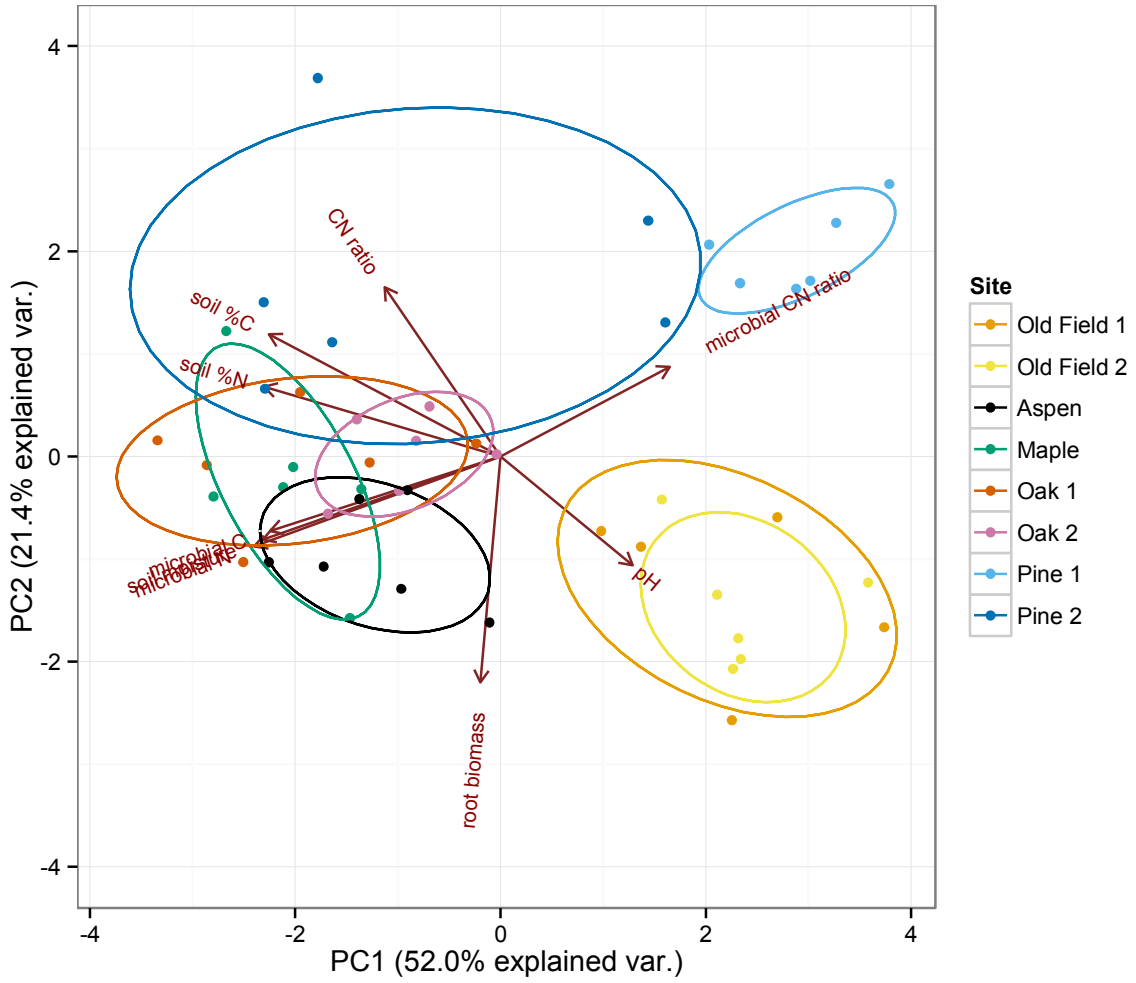


Figure 2.1. PCA of measured site characteristics just from ambient plots (PC 1 explains 52.0% of variation, PC 2 explains 21.4% variation).

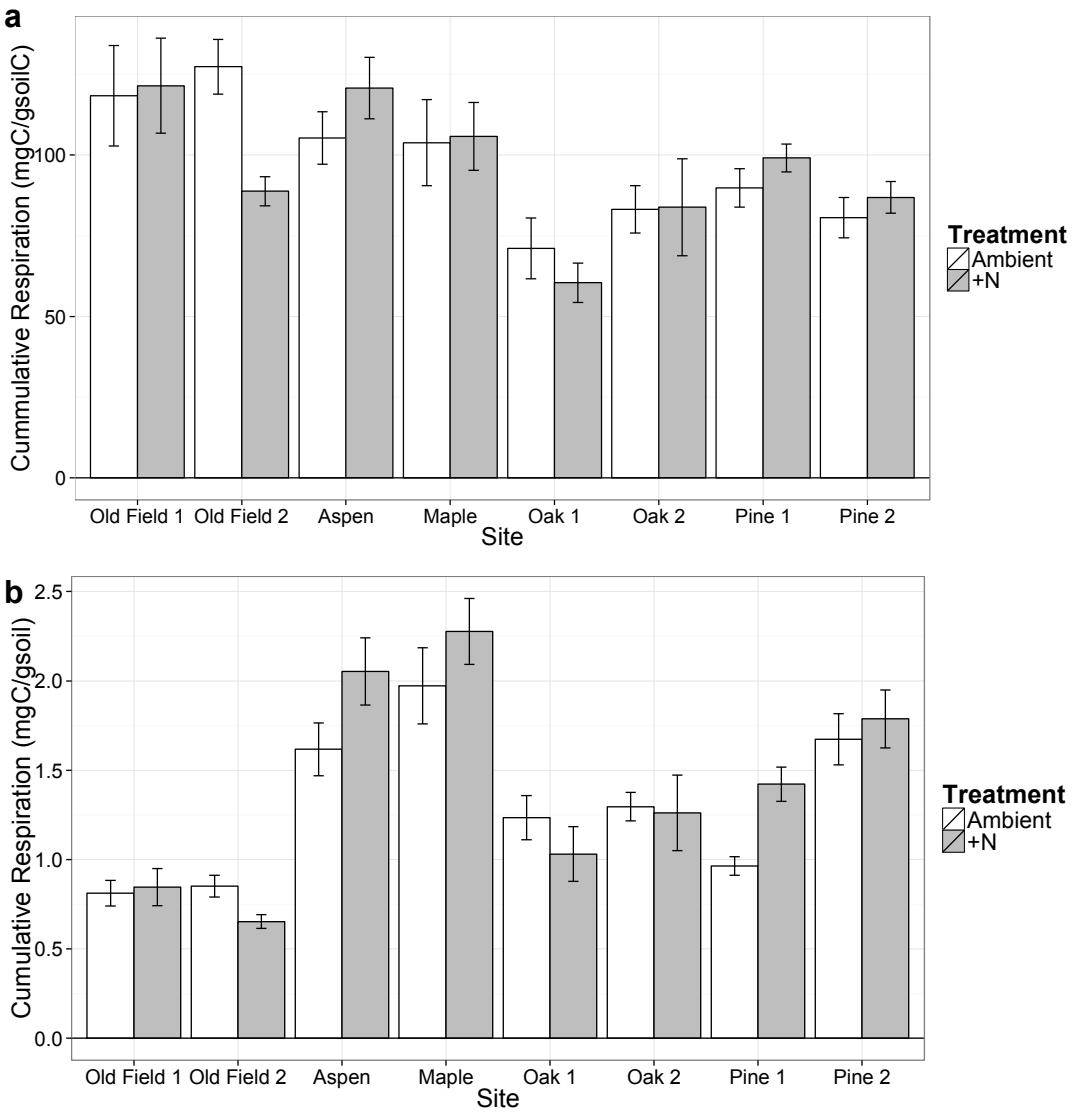
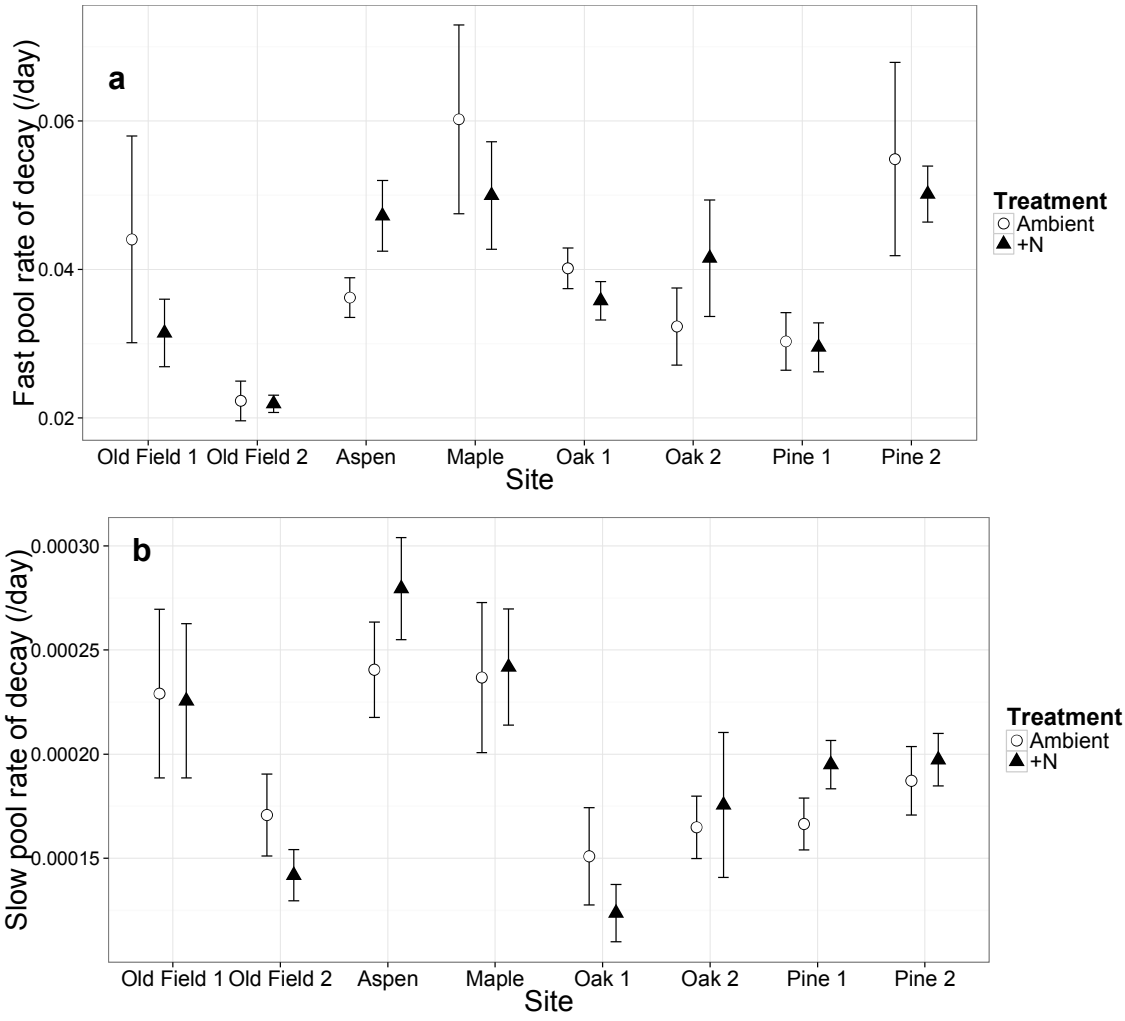


Figure 2.2. Cumulative microbial respiration over the duration of the incubation by site and N treatment, expressed a) per gram soil C, and b) per gram soil. Respiration per gram soil C did not differ by N treatment (N Treatment $P = 0.6845$; N*Site interaction $P = 0.2047$), but did differ significantly between sites ($P = 6.188e-08$). The response of respiration per gram soil to N addition depended on site (N*Site interaction $P = 0.02042$), and overall differed significantly between sites ($P<2e-16$). Original data are shown, although statistics were run using log-transformed data to validate model assumptions.


a
Fast pool rate of decay (/day)
0.06
0.04
0.02
Old Field 1
Old Field 2
Aspen
Maple
Oak 1
Oak 2
Pine 1
Pine 2
Site
Treatment
Ambient
+N
b
Slow pool rate of decay (/day)
0.00030
0.00025
0.00020
0.00015
Old Field 1
Old Field 2
Aspen
Maple
Oak 1
Oak 2
Pine 1
Pine 2
Site
Treatment
Ambient
+N

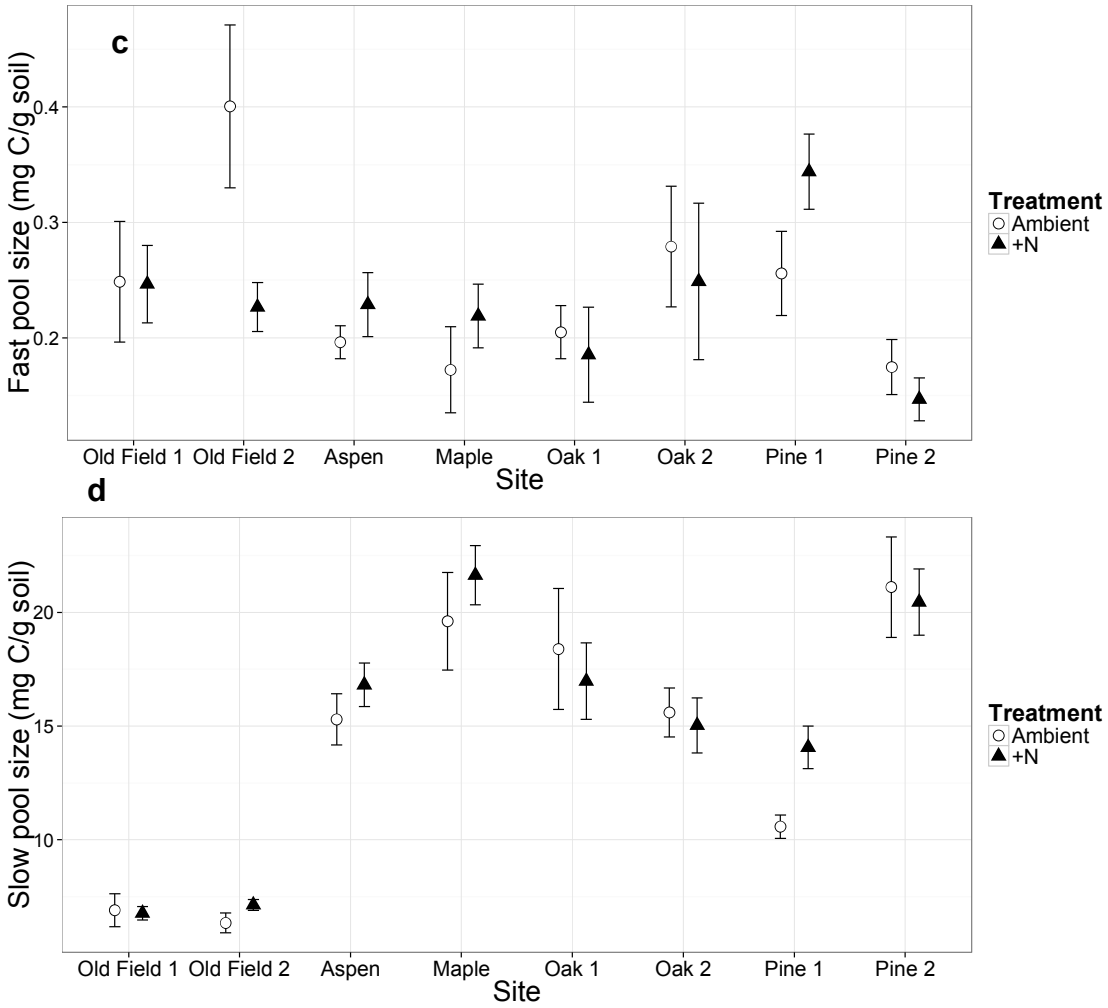


Figure 2.3. Carbon pools and decay rates by site and N treatment: a) Fast pool decay rate (k_f); b) slow pool decay rate (k_s); c) fast pool size (C_f); d) slow pool size (C_s). Original data are shown for ease of interpretation, however k_f, k_s, and C_s were log-transformed for statistical analysis to meet model assumptions.

CHAPTER 3

Elevated CO_2 and N addition alter rhizosphere priming of soil organic matter decomposition

Abstract

As the largest terrestrial pool of carbon (C), even relatively small changes in soil C could have large implications for atmospheric carbon dioxide (CO_2) concentrations. Rhizosphere priming of soil organic matter decomposition is an important source of soil C loss, and could respond to changing environmental conditions. We tested the effects of elevated CO_2 and N addition on soil CO_2 flux and the amount of CO_2-C specifically released from soil by rhizosphere priming of soil organic matter decomposition. We found a positive feedback loop; higher atmospheric CO_2 levels caused higher rates of decomposition of soil organic matter and thus CO_2-C emission. To do this, we used a mesocosm study within the Biodiversity, CO_2, and Nitrogen Experiment at the Cedar Creek Ecosystem Science Reserve in central Minnesota, USA. We harnessed differences in stable isotope chemistry between plants and soil (based on C3/C4 differences) to partition the amount of total CO_2 originating from SOM, and the specific amount decomposed as a result of rhizosphere processes. Overall, elevated CO_2 increased the soil C loss from rhizosphere priming of decomposition by 34-39% on average (on a per g soil and per g soil C basis, respectively), and N addition reduced the soil C loss from priming by 29% on average (on a per g soil C basis, there was no N effect on the RPE when

expressed per g soil). We also found substantial differences in the amount of C lost from rhizosphere priming of decomposition between the two C3 grass species studied – either 340% or 460% depending on the metric of C flux used (on a per g soil and per g soil C basis, respectively). Finally there was no mediation of the elevated CO_2-induced rhizosphere priming effect with N addition (i.e. no CO_2*N interaction), as we expected if plant nutrient status is a primary driver of the rhizosphere priming CO_2 effect. These findings support the hypothesis that increasing concentrations of CO_2 could result in greater loss of old, SOM-derived C from grassland soils.

Introduction

How terrestrial ecosystems will contribute to carbon (C)-climate feedbacks under environmental change will depend on how soil C stocks respond. Globally, soils are the largest terrestrial pool of C – presently holding 2-3 times more C than all standing biomass around the world and 3-4 times more C than is present in the atmosphere (Ciais *et al.*, 2013). Even proportionally small changes in soil C could therefore have large consequences for atmospheric carbon dioxide (CO_2) levels. However, how this C stock will respond to an increasingly altered global environment, one characterized by rising concentrations of atmospheric CO_2 (Friedlingstein *et al.*, 2014) and nitrogen (N) deposition (Galloway *et al.*, 2008), remains uncertain.

An important loss pathway for old soil C, that could be sensitive to global change, can occur through rhizosphere priming (Cheng *et al.*, 2014; Finzi *et al.*, 2015). Fresh plant inputs of low-molecular weight C compounds can stimulate microbial activity in the rooting zone, or rhizosphere, resulting in an increase (or decrease) in decomposition of nearby soil organic matter (SOM) (Kuzyakov *et al.*, 2000; Kuzyakov, 2010; Cheng *et al.*, 2014). This rhizosphere priming effect (RPE) – defined in the literature as the difference in SOM decomposition from root and rhizosphere processes compared to root-free soil – can lead to a range of outcomes from a 50% decrease in soil C loss to a 380% increase (Cheng *et al.*, 2014). The RPE has been shown to positively relate to plant productivity (Dijkstra *et al.*, 2006), where greater growth can result in more C inputs belowground and hence greater release of soil C from rhizosphere priming. Additionally, the RPE can

increase when plants are more N-limited (Dijkstra, 2013), where greater plant investment of C resources belowground into roots and increased exudation can result in enhanced soil organic matter decomposition and liberation of N that can then be taken up by plants (Dijkstra *et al.*, 2009). However, despite the growing recognition of the importance of rhizosphere priming in soil C loss and N mineralization, as well as the mechanisms involved, it less clear how the RPE might respond to global change (Cheng *et al.*, 2014).

Because CO_2 and N are plant resources that affect growth and nutrient demand, change in their availabilities could influence the magnitude of the RPE. As anthropogenic CO_2 emissions continue to rise, plant growth will become increasingly limited by nutrients (Reich & Hobbie, 2013), likely resulting in greater plant investment belowground into roots and mycorrhizal symbionts to improve access to soil nutrients (Zhu & Miller, 2003; Johnson, 2010). This increase in belowground C allocation and rhizodeposition, or the release of root-related C (Pendall *et al.*, 2004; Adair *et al.*, 2009), could result in greater C loss from rhizosphere priming (Cheng & Johnson, 1998). However, the size of the RPE increase at elevated CO_2 could depend on availability of soil nitrogen. If plants are N-limited under elevated CO_2 there could be enhanced exudation as a means to access more N through increased decomposition (Dijkstra *et al.*, 2008; Drake *et al.*, 2011). However, this positive RPE might decrease at higher N availability, if plants invest less C belowground (Phillips *et al.*, 2011). But while several experiments have addressed the effect of elevated CO_2 on the RPE, few have examined how the CO_2-induced C loss might change with N availability (Dijkstra, 2013; Cheng *et al.*, 2014). Given

simultaneous increases in CO_2 emissions and N deposition, as well as the sizable current and potential contribution of the RPE to soil C loss, determining the RPE in response to both elevated CO_2 and N availability is particularly important for understanding future C cycling.

Our objective was to test how elevated CO_2 and increased N deposition influence the fate of already-stored C in soils, and whether the responses differ when different plant species are planted in the soil. We hypothesized that the RPE would increase under elevated CO_2, decrease with N addition, and not change when exposed to both elevated CO_2 and N addition, following the rationale that plants would alter allocation of resources belowground and stimulate a positive RPE to liberate more N when N was limiting. We included two species of C3 grasses as test cases for our main question. Given the role of plant biomass in determining the RPE (Dijkstra *et al.*, 2006), we expected slight differences between the two species in the magnitude of their associated RPE, but that overall the patterns in their RPE responses to CO_2 and N would be similar. In addition, we expected the magnitude of the RPE would further relate to three key plant and soil characteristics: 1) total plant biomass (as indicated above); 2) soil C:N as an indicator of N availability, where priming would be higher at higher soil C:N ratios; and 3) activity of arbuscular mycorrhizal fungi (AMF), where we expected greater priming with more AMF activity. AMF are symbiotic plant fungi common in grasslands that provide nutrients to the plant hosts in exchange for nutrients. AMF have recently been shown to release soluble C to rhizosphere microbial communities (Kaiser *et al.*, 2014), which could

serve as a way to prime soil organic matter decomposition and liberate inorganic N to transfer to the host plant under low-N conditions (Talbot *et al.*, 2008), as was recently shown with litter decomposition (Cheng *et al.*, 2012). They also have been shown to increase in abundance under elevated CO_2 (Antoninka *et al.*, 2011).

We tested our hypotheses through an experiment that leveraged natural variation in soil and plant stable isotope chemistry to test the effects of elevated CO_2 and N addition on the RPE in mesocosms planted with two common C3 grassland species. Grasslands are particularly important systems for studying C cycling dynamics with global change as grasslands cover 30-40% of the ice-free land globally (Foley *et al.*, 2007) and hold a large portion of Earth's total soil C (Jobbágy & Jackson, 2000). Furthermore, with robust root systems and the majority of biomass underground, rhizosphere priming could be a particularly important mechanism of C loss in grassland soils.

Materials and Methods

We designed a mesocosm study to harness differences in soil and plant stable C isotopes, taking advantage of the $\delta^{13}C$ signal in elevated CO_2 experiments and C3/C4 vegetation differences. Stable isotopes provide a valuable tool for C cycling research (Amundson & Baisden, 2000), particularly for partitioning total soil CO_2 flux into its component sources to quantify the RPE (Pendall *et al.*, 2003). Specifically, we used plants with a relatively depleted C signature, due to having the C3 photosynthetic pathway, and planted them in soils with a more enriched C signature (from being historically dominated by C4 plants).

This experimental approach provided a way to distinguish recent plant-derived C inputs from C substrates in the soil.

Study Site

We set up the study as a sub-experiment in pots within the full Biodiversity, CO_2, and N (BioCON) Experiment at the Cedar Creek Ecosystem Science Reserve in central Minnesota, USA (Lat. 45N, Long. 93W; Reich *et al.*, 2001). The CO_2 treatment at BioCON is applied using a Free Air CO_2 Enrichment (FACE) system using ^{13}C-depleted CO_2, where the three ambient rings receive ambient air and the three elevated rings receive ambient +180ppm CO_2. We also mimicked the N treatment of the full experiment (+4 g N m^{-2} y^{-1} as NH_4NO_3), described below. Pots were set up within the six FACE rings: in each ring, pots were randomly arranged into two trays that sat in one large plastic container (that collected excess water to avoid watering other experimental plots) in one of the ring's bare ground plots (which had been randomly assigned to a location among the experimental plots in BioCON's establishment). Over the time of the study (mid May – late August, 2015), the average daily highs were 24.9°C and the average daily lows were 13.0°C. There was a total of 696.7mm of rainfall during the period.

Experimental Design

Because our initial intent was to test the effects of AMF on the RPE under global change, we grew plants from seed in sterilized soil with a full factorial of CO_2, N, and AMF treatments, which were randomly assigned after germination (N=6 for each treatment

combination, for a total N=96 planted samples). We also established unplanted controls to understand the $\delta^{13}C$ signature and quantity of microbial SOM decomposition end member.

We harnessed natural variation in stable C isotopes as a way to trace C input sources. Due to greater fractionation against the heavier ^{13}C isotope during C3 photosynthesis, C3 plants tend to have lower (more negative) $\delta^{13}C$ values compared to C4 plants. To create a strong enough contrast in stable C isotopes of the plants and soil in order to use ^{13}C as a tracer, we therefore used C3 plants with a relatively ^{13}C-depleted signature and grew them in pots with soils that had long been dominated by C4 plants and were relatively enriched in ^{13}C. We used two C3 grass species, *Bromus inermis* and *Agropyron repens*, and grew each in monoculture (to avoid complications for building and interpreting mixing models). Both *Bromus inermis* and *Agropyron repens* are highly mycorrhizal (Antoninka *et al.*, 2011) and abundant in BioCON. Seeds were surface-sterilized (using 10% household bleach, with the main acting agent Sodium hypochlorite, NaClO). Seeds were planted in a D40H Deepot (6.4cm in diameter by 25.4cm in depth, 656 ml volume, Stuewe & Sons, OR, USA) filled with ^{13}C-enriched soil harvested from a long-term C4 grass-dominated area at Cedar Creek (and previously analyzed for $\delta^{13}C$) which had been homogenized, sieved through a 2mm sieve, and sterilized at 180°C for two 24 hour periods (mixed in between) to eliminate microbial activity (Endlweber & Scheu, 2006). The Cedar Creek soils used for the experiment are nutrient-poor, established on sandy outwash plain (>90% sand), and designated as Udipsamments (Grigal, 1974).

We used a N treatment that was about a ten-fold increase over current ambient N deposition at Cedar Creek (according to the National Atmospheric Deposition Program). The rate of application in this study (+10.6 g N m^{-2} y^{-1}) was higher than the +N application rate for the full BioCON experiment (+4 g N m^{-2} y^{-1}), but in the range of what others have used for studying N effects on soil C cycling (Frey *et al.*, 2014). Half of the pots received N as a diluted NH_4NO_3 solution over the growing season and the other half received no added N, only water applied in equal amounts as the water in the +N treatment. Each time we watered we fertilized each of the +N pots with 0.002g N per pot in the form of diluted NH_4NO_3 with 40ml of water. Non-fertilized, ambient N pots received 40ml of water. We watered/fertilized about every other day, depending on rain.

We applied two microbial treatments to the sterilized soil, one intended to include AMF and one intended to exclude, or highly reduce, AMF. We created inoculants for each treatment from the same Cedar Creek soil used for the experiment (following Koide & Li, 1989). Briefly, we combined soil with water to create a slurry following the ratio of 100g of soil to 300ml. For the more inclusive treatment (*INC*), we filtered the slurry through a 250µm sieve with the intent of excluding most large soil components or soil animals. For the more restricted treatment (*REST*), we filtered the slurry through a 53µm sieve with the intent of excluding most AMF spores (Koide & Li, 1989). Pots were drenched with 50ml of the appropriate inoculant after seedlings germinated, and then again 3-4 weeks later. The mycorrhizal treatment was intended to manipulate presence,

but may have had as large an effect on AMF composition. The *REST* treatment likely reduced presence of *Gigaspora* mycorrhizae whose spores tend to be larger than 53µm (or can even be larger than 250µm), therefore potentially providing a competitive advantage to *Glomus* mycorrhizae whose spores are often smaller than 53µm (*N. Johnson personal communication*). Because we cannot clearly interpret the microbial treatment (and because it had few significant effects on C cycling and plant and soil characteristics, see below), we focused our analysis and interpretation on the effects of plant species and global change treatments and do not discuss the *INC*/*REST* treatments further.

Finally, in addition to the planted pots, we also established unplanted pots (sterilized soil across microbial*N treatment combinations) to determine the $\delta^{13}C$ of the SOM decomposition end member for use in isotopic mixing models to quantify SOM decomposition in the absence of plants and calculate priming (N=24; see below for more detail in *Isotopic Mixing Models and Priming Calculations*). Because there were no plants in these pots we expected no difference by CO_2 treatment, and therefore only had unplanted controls in the ambient CO_2 rings to cut down on sample number.

Sample Collection and Analysis

Intact and undisturbed pots, with their plants, were brought back to the lab to measure CO_2 efflux for both concentration and isotopic signature. Pots (both planted and unplanted) were put into small plant chambers (Fig. S1, after Dijkstra *et al.*, 2010), CO_2 was scrubbed down using a vacuum system with soda lime, and chambers were covered

by impermeable black plastic for 90 minutes (or longer if the CO_2 concentration was below the detection limit for $\delta^{13}C$). Gas samples were then taken from the chamber with a syringe to assess CO_2 concentration on an infrared gas analyzer (LI-COR, Lincoln, NE, USA). This total CO_2 flux therefore includes soil respiration, root respiration, and shoot respiration. Gas samples were simultaneously taken and stored in evacuated Exetainer vials to later measure $\delta^{13}C$ of CO_2 (GasBenchII connected to a ThermoFinnigan DeltaV).

Pots were then destructively sampled to isolate and measure plant and soil isotopic components and other potentially relevant covariate data. Plant shoots were separated from roots, dried, and ground. Roots (including crowns) were separated from soil, then roots were washed, dried, and ground. Ground shoots and roots were each analyzed for %C, %N and $\delta^{13}C$ and ^{15}N using an EA-IRMS (Costech 4010 Elemental Analyzer coupled to a Thermo Delta Plus XP IRMS). Soil from pots was sieved through a 2mm sieve subsampled for gravimetric water content (dried at 105°C for at least 24 hours), phospholipid fatty acid analysis (stored immediately at -80°C), and total microbial biomass (using the chloroform fumigation technique, Brookes *et al.*, 1985)), and the remaining soil was then air-dried. A portion of the air-dried soil from each pot was used to measure pH using a 2:1 water:soil method (Hendershot et al. 1993), and a portion was ground and analyzed for %C, %N and $\delta^{13}C$ and ^{15}N using an EA-IRMS (Costech 4010 Elemental Analyzer coupled to a Thermo Delta Plus XP IRMS).

Microbial Lipid Extraction

We used phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) analysis to assess treatment effects on AMF abundance, as well as non-mycorrhizal fungi and bacteria. Phospholipids are present in both bacterial and fungal cellular membranes, whereas neutral lipids are only present in fungi (which produce them as storage lipids). Specific lipids (both phospho- and neutral lipids) are produced by unique fungi and bacteria so can serve as indicators of the abundance of these organisms. Following a modified Bligh and Dyer (1959) method, described in detail by (Herman *et al.*, 2012), we extracted, isolated, and quantified abundance of lipid biomarkers characteristic of certain fungi and bacteria. We retained the NLFAs and analyzed them separately as another way to assess activity of general and arbuscular mycorrhizal fungi (Olsson *et al.*, 1995; Schmidt *et al.*, 2017). Briefly, 10 g of freeze-dried soil were extracted three times using a mixture of chloroform (6ml), citrate buffer (5ml), and methanol (12ml). We left phases to separate overnight at room temperature, and then removed the aqueous phase by aspiration and reduced the chloroform phase using a RapidVap evaporator. We separated lipid classes using silica column chromatography and then converted extracted phospholipids and neutral lipids to fatty acid methyl esters using a milk alkaline methylation procedure (Schmidt et al. 2017), followed by extraction using a 30µl Hexane dilution. Methyl-esterfied fatty acids were analyzed (2µl injection) on an Agilent 7890 Gas Chromatograph coupled to an Isoprime 100 Isotope Ratio Mass Spectrometer. We converted lipid peak areas to nmol lipid / g dry soil using a 13:0 internal standard added to each sample and also analyzed separately.

We focused our analysis of lipids on key biomarkers of arbuscular mycorrhizal fungi and broad fungal and bacterial groups. Specifically, we tested treatment effects on the lipids commonly produced by *Glomus* arbuscular mycorrhizal fungi (16:1ω5 phospho- and neutral lipids (Wilkinson *et al.*, 2002; Gutknecht *et al.*, 2012a), although gram negative bacteria also produce the phospholipid; *Gigaspora* arbuscular mycorrhizal fungi, common plant colonizers at Cedar Creek (Ji *et al.*, 2013), and general fungi (18:1ω9c phospho- and neutral lipids, Graham *et al.*, 1995); other non-mycorrhizal fungi (18:2ω6,9c; Balser & Firestone, 2005; Gutknecht *et al.*, 2012b); gram positive bacteria (15:0 iso, Gutknecht et al. 2012, Wilkinson et al. 2002); and gram negative bacteria (16:1ω7c, Gutknecht et al. 2012, Wilkinson et al. 2002). We also assessed biomarkers for total microbial biomass (for which we used a subset of key lipids based on analysis performance: 14:0, 15:0 anteiso, 15:0 iso, 16:0, 16:0 10me, 16:1ω5c, 16:1ω7c, 16:1ω9c, 18:0, 18:0 10me, 18:1ω9c, 18:1ω9t, 18:2ω6,9c). However, this metric of total microbial biomass did not correspond closely to the microbial biomass determination from the chloroform fumigation method (R^2=0.0528, P=0.0213), perhaps due to using just a subset of lipids as described above.

Isotopic Mixing Models and Priming Calculations

We used a two-part mixing model to partition the total CO_2-C flux into the flux from SOM-C and the flux from recent plant-derived C inputs (either root respiration or microbial decomposition of recent plant inputs, like root exudates or sloughed-off root fragments, although we were not able to distinguish between these two sources). From

the main mixing model (*Eq. 1*), assuming that the total amount of respired CO_2-C (C_{CO2}) is comprised solely of plant-derived CO_2-C (C_{plant}) and SOM-derived CO_2-C (C_{soil}), we can rearrange *Eq. 2* and substitute into *Eq. 1* to solve for the proportion of total CO_2-C from soil, shown in *Eq. 3*. We then multiplied the proportion of soil-derived CO_2-C by the total CO_2-C respired to get the amount of C respired specifically from SOM (*Eq. 4*).

Equation 1 $(\delta^{13}C_{CO2} * C_{CO2}) = (\delta^{13}C_{plant} * C_{plant}) + (\delta^{13}C_{soil} * C_{soil})$

Equation 2 $C_{CO2} = C_{plant} + C_{soil}$

Equation 3 Proportion soil CO_2-C $= C_{soil} / C_{CO2} = (\delta^{13}C_{plant} - \delta^{13}C_{CO2}) / (\delta^{13}C_{plant} - \delta^{13}C_{soil})$

Equation 4 $C_{soil} = C_{CO2} (\delta^{13}C_{plant} - \delta^{13}C_{CO2}) / (\delta^{13}C_{plant} - \delta^{13}C_{soil})$

For these mixing models we used measurements from each individual sample for $\delta^{13}C_{CO2}$ and $\delta^{13}C_{plant}$, which specifically came from root tissue. For the $\delta^{13}C_{soil}$ we used an average $\delta^{13}C$ value from CO_2 produced from soil organic matter decomposition in unplanted pots (N=24). Unplanted pots were exposed to microbial and N treatments, but showed no treatment difference in $\delta^{13}C$ ($P>0.1$) so we averaged across all samples for simplicity. ($\delta^{13}C$ values for all plant, CO_2, and soil components are reported in Table S1.)

To quantify the amount of SOM-derived C released specifically from rhizosphere priming, we took the total SOM-derived CO_2-C flux (C_{soil}) and subtracted the C flux from the average microbial decomposition of SOM in unplanted pots ($C_{unplanted}$) receiving

similar conditions (*Eq. 5*).

Equation 5 $C_{primed} = C_{soil} - C_{unplanted}$

The unplanted pots received the full factorial of N*microbial treatments (N=5 or 6 for each of the four sets of N*microbial treatment combinations) so we were able to subtract the average microbial decomposition from pots experiencing the same conditions to those of the individual sample. We did not include CO_2 treatments for these unplanted controls because CO_2 should only influence soil processes through its effects on plants.

Statistical Analyses

We used linear mixed effects models to test the effects of elevated CO_2, N addition, plant species, and the microbial treatment on decomposition of older SOM. For all response variables, we evaluated models that included main effects, two-way, three-way, and four-way interactions of CO_2, N, species, and microbial treatments as fixed effects and CO_2 nested in Ring as a random effect. In addition to treatment-only models, to further understand priming responses we also built models that included key covariates related to our hypotheses: total plant biomass, soil C:N, and abundance of the *Glomus* AMF-associated neutral lipid (16:1ω5c) (covariates were not strongly correlated with other metrics, except total plant biomass which was highly related to both shoot and root biomass; Table S2). There was only one significant covariate*treatment interaction when assessed on a per g soil C basis (16:1ω5c neutral lipid abundance*Species, P=0.0170),

and the covariate interaction model and the no-interaction covariate models were only marginally significantly different (P=0.0748), so we left out the lipid*Species interaction for simplicity. Additionally, we were primarily interested in CO_2 and N effects, and the lipid*Species interaction did not substantially change the RPE response to those treatments. The covariate interaction and no-interaction models were also not different for the RPE when expressed on a per g soil basis, so for both metrics the covariate models included main effects of the three covariates in the broader model along with all the main effects, two-, three-, and four- way interaction effects of CO_2, N, species, and microbial treatments. Although the microbial treatment was included in models for statistical completeness, for the sake of simplicity, and because the treatment had very few main effects on response variables, we do not discuss it further here. Response variables were natural log-transformed, square root-transformed, or arcsin-square-root transformed (in the case of proportions) to meet model assumptions of normality and equal variance. All analyses were done in R (R Core Team 2013). We used the lmer function in the lme4 package in R to run models (Bates *et al.*, 2015) and the piecewiseSEM package (Lefcheck, 2015) to determine marginal and conditional R^2 values.

Results

Plant and soil characteristics

Plant biomass growth and allocation responded to elevated CO_2 and N addition, sometimes in different ways depending on the plant species (Tables 1 and S3). The two

plant species grew slightly differently, both in terms of total growth and shoot:root ratios. *Bromus inermis* grew, on average, about double the shoot biomass and about four times the root biomass, and therefore had a lower shoot:root ratio, than *Agropyron repens* ($P<0.0001$ for all responses). Overall, shoot biomass was higher (by 27% on average) under elevated CO_2 ($P=0.0062$), as was root biomass (by 14% on average) ($P=0.0503$), as we expected. Combined, total plant biomass was about 19% greater under elevated CO_2 ($P=0.0144$). There was no effect of N addition on shoot biomass ($P>0.1$) but there was a Species*N interactive effect for root biomass, where N addition resulted in slightly lower overall root biomass in *B. inermis* ($P=0.0495$; Table S3). There was relatively more investment in shoots than roots (a higher shoot:root ratio) in *B. inermis* under N addition (Species*N $P=0.0078$, Table S3).

In addition to changes in overall biomass, plant tissue chemistry also responded to global change treatments. Shoot %C did not vary much, ranging on average from 41.7 to 43.2%. However, shoot %N differed between the two species, with N addition, and there was a Species*N interaction ($P=0.0049$, $P<0.0001$, and $P<0.0001$, respectively; Table S3): ambient shoot %N was higher in *A. repens* than in *B. inermis*, and overall shoot %N was higher with N addition, but especially so in *B. inermis*. Root %C overall was slightly lower in *B. inermis* than in *A. repens* ($P=0.0002$). Root %N, overall, was slightly higher with N addition, but mostly due to the positive response in *B. inermis* (N $P=0.0040$ and Species*N $P=0.0122$, respectively; Table S3).

Some of the soil characteristics relevant for understanding C cycling also differed by treatments (Tables 2 and S4). Soil moisture was generally lower in pots with *B. inermis*, compared to pots with *A. repens* ($P<0.0001$), likely due to larger plant shoot and root biomass as noted above. Soil moisture was also slightly higher with N addition ($P=0.0495$), although this was mostly driven by the response in *A. repens* (Species*N interaction $P=0.0405$). In contrast, soil pH did not differ by treatments ($P>0.1$), and ranged from 5.82-6.96, with an average across treatments of 6.48 (Table S4). Both soil %C and %N were higher under N addition (P>0.0001 for both, Table S4 and Fig. S2), and soil C:N ratio was lower with N addition ($P=0.0250$).

Soil microbial responses

Microbial biomass characteristics were less responsive to elevated CO_2, but did differ between species and with N addition (Tables 2 and S4). Microbial biomass C and N, determined by chloroform fumigation, were higher in *B. inermis* than in *A. repens* ($P=0.0081$ and $P<0.0001$, respectively). As we expected, microbial biomass N was also higher under N addition ($P=0.0481$). The microbial biomass C:N ratio had a significant Species*CO_2 interaction ($P=0.0277$). In contrast, total microbial biomass, as determined by PLFA analysis, did not respond much to global change treatments (Table 3). In addition to total microbial biomass, however, in order to develop a more detailed view of soil C cycling and who is involved, we also tested the responses in key groups of microbes: arbuscular mycorrhizal fungi, general fungi, and bacteria.

Mycorrhizal phospho- and neutral lipid biomarkers differed by global change treatments, indicating responses in both abundance and physiology of these plant symbionts. Abundance of the 16:1ω5c phospholipid, commonly found in the cell membrane of mycorrhizal fungi, particularly *Glomus* (Graham *et al.*, 1995), as well as gram negative bacteria, was lower in *B. inermis* (Species $P<0.0001$, Table 3). There were also significant interaction effects of CO_2*N ($P=0.0455$). The 16:1ω5c neutral lipid, a storage lipid found in mycorrhizal fungi, particularly *Glomus*, also responded to treatments, indicating changes in activity of these organisms: the response to CO_2 differed by species (Species*CO_2 $P=0.0177$). The 18:1ω9c neutral lipid associated generally with fungi, but also common in *Gigaspora* mycorrhizae (Graham *et al.*, 1995), also had a significant Species*CO_2 interaction ($P=0.0443$), where elevated CO_2 increased 18:1ω9c abundance most in *B. inermis*.

Non-mycorrhizal fungal and bacterial biomarkers also responded to treatments (Tables 3 and S4). Abundance of the 18:2ω6,9c phospholipid, a general fungal biomarker, differed between the plant species and there was a significant CO_2*N interaction ($P=0.0462$ and $P=0.0454$, respectively). The neutral lipid, however, did not respond to any treatments ($P>0.1$). The 15:0 iso phospholipid, an indicator of gram positive bacteria, was overall lower in *B. inermis* and in ambient N pots (Species $P=0.0114$, N $P=0.0219$). The 16:1ω7c phospholipid, a good indicator of gram negative bacteria, did not respond to global change treatments ($P>0.1$).

Total and partitioned CO_2 flux responses to treatments

The total amount of CO_2-C respired from pots, which included microbial respiration of recent plant-derived C and SOM-derived C, as well as root and shoot respiration, differed between plant species and with global change treatments (Table 4). On a per g soil basis, CO_2-C released from pots was greater from *B. inermis* pots than from *A. repens* pots (P<0.0001). The total CO_2-C flux was also greater overall under elevated CO_2 (P=0.0147; Fig. 1a), by 21% on average. On a per g soil C basis, fluxes were also higher from *B. inermis* pots and under elevated CO_2, by 25% on average (Species P<0.0001 and CO_2 P=0.0138). Total CO_2-C flux (on per g soil C basis), was lower with N addition, on average by about 8% (P=0.0138; Fig. 1b).

There were no significant effects of elevated CO_2 or N addition on the proportion of flux from SOM-derived C, but the trend was towards lower proportions of total flux coming from soil with global change treatments, on average (CO_2 P=0.0680 and N P=0.0984; Fig. 3). The proportion of total respired CO_2-C from soil C, as determined by the mixing model described above (Eq. 1), was on average about 46% with *B. inermis* and 67% with *A. repens* (Species P<0.0001). However, the total amount of SOM-derived C respired did differ between plant species and by global change treatments (Table 4 and Fig. S3). This SOM-derived C flux, which represents both ambient microbial decomposition of SOM (in the absence of root effects) and priming-related decomposition of SOM, was greater with *B. inermis* and under elevated CO_2 when expressed on either a per g soil or per g soil C basis (mg C/hr/g soil: Species P<0.0001 and CO_2 P=0.0213; mg C/hr/g soil C:

Species $P<0.0001$ and CO_2 $P=0.0189$). In contrast, the total amount of SOM-derived C released was lower with N addition (mg C/hr/g soil: N $P=0.0375$; mg C/hr/g soil C: N $P<0.0001$).

Rhizosphere priming effect

The amount of SOM-derived C released from rhizosphere priming across all our samples ranged almost ten-fold, from slightly negative values (indicating less respiration of SOM-derived C with plants present than without plants) to more positive values. We again found effects of plant species, elevated CO_2, and N addition (Table 4 and Fig. 2). The total amount of C released from the RPE, both when expressed on a per g soil and per g soil C basis, was greater in *B. inermis* and with elevated CO_2 (mg C/hr/g soil: Species $P<0.0001$ and CO_2 $P=0.0213$; mg C/hr/g soil C: Species $P<0.0001$ and CO_2 $P=0.0490$). The effect of N addition was only significant when the amount of C lost from the RPE was expressed per g soil C, in which case primed C was lower with N addition (mg C/hr/g soil C: N $P=0.0030$).

Rhizosphere priming effect responses were partially explained by covariates, but not entirely (Table 5). After including plant and soil covariates (soil C:N ratio, total plant biomass, and 16:1ω5c neutral lipid abundance) in the model, the amount of C lost from rhizosphere priming (on a per gram soil basis) was still greater overall with elevated CO_2 and under *B. inermis* compared to *A. repens* (CO_2 $P=0.0233$ and Species $P<0.0001$). This model, however, did not explain more of the variation than the treatment-only model

(Table 4), and the covariates were not actually significant (except 16:1ω5c neutral lipid abundance, which was marginally significant, and tended to correspond with an increased RPE: P=0.0778). When expressed per gram soil C, the covariate model explained about 10% more variation than the treatment-only model, and also provided more insight into what might be contributing to treatment responses. The positive CO_2 effect on the RPE (expressed as mg C/hr/g soil C) became less significant when plant and soil covariates (soil C:N ratio, total plant biomass, and 16:1ω5c neutral lipid abundance) were included in the model (CO_2 P=0.0686). Although there may still be some additional effect of elevated CO_2 on the RPE, this suggests that much of the increase in SOM-derived C flux from rhizosphere priming at elevated CO_2 may be because plants are also larger (Total plant biomass P=0.05723; Tables 1 and S3 and Fig. S6), and likely thus producing more rhizodeposits. The effect of N addition was still significant after including covariates (N P<0.0001), which shows that regardless of other plant and soil characteristics, the more N added to the system, the smaller overall RPE. Similarly, the amount of primed C also differed between species after including covariates (Species P=0.0010), suggesting that there may be something unique about the types of rhizodeposits the two species produce that differentially stimulate C loss from rhizosphere priming (Fig. S5). Contrary to our expectation and to the N treatment effect, there was actually a smaller RPE and less C lost at higher soil C:N (when N was relatively less available) (Soil C:N P<0.0001, Fig. S6). There was also a positive effect of 16:1ω5c neutral lipid abundance (16:1ω5c P=0.0073; Fig. S6), the biomarker for arbuscular mycorrhizal fungi, and *Glomus* species in particular (Graham *et al.*, 1995).

Overall, the percent of the total CO_2-C flux that came specifically from rhizosphere priming release of SOM-C ranged from 0 to 39% (priming made no contribution to total flux in samples with negative priming). On a per g soil basis, this contribution of rhizosphere primed C to the total CO_2 flux was higher in *B. inermis* and also generally higher under elevated CO_2 ($P<0.0001$ and $P=0.0327$, respectively), although there was a Species*CO_2 interaction as well that suggested the increase in proportion of primed C with elevated CO_2 was greater in *A. repens* ($P=0.0055$; Fig. S4a). The contribution of primed C towards the CO_2 total flux when assessed on a per g soil C basis also was higher overall in *B. inermis* and a Species*CO_2 interaction again suggested that the proportion of primed C increased with elevated CO_2, but only in *A. repens* (Species $P<0.0001$ and Species*CO_2 $P=0.0249$, respectively; S4b).

Discussion

Overall, we found partial support for our hypotheses. Indeed there was greater rhizosphere priming of SOM decomposition (a larger RPE), and overall increases in CO_2 flux, under elevated CO_2 and less rhizosphere priming of SOM with N addition (a smaller RPE). However, we had also expected a CO_2*N interaction where the RPE is enhanced under elevated CO_2, but not when N is also added, which we would expect if rhizosphere priming of SOM decomposition can be plant-regulated as a means to liberate more N during periods of limitation. There was no evidence for this mediating effect of N addition on CO_2-induced priming. We also found differences between the RPE under the

two C3 grass species that could not be explained by species difference in biomass alone. Here we discuss possible reasons for the global change and species differences in the observed RPE, as well as implications of these findings.

Effects of elevated CO_2 and N addition on the RPE

The observed responses in the RPE to global change generally agree with what others have found, and our expectations. The increase we observed in the RPE with elevated CO_2 was on average 34% when expressed per g soil and 39% when expressed per g soil C. A positive effect of elevated CO_2 on RPE has been found before (Cheng & Johnson, 1998; van Groenigen *et al.*, 2014), but a recent meta-analysis reported no overall effect of elevated CO_2 on old soil C release (although perhaps due to low sample size; (van Groenigen *et al.*, 2017). Here, the response to elevated CO_2 is mostly, but not entirely explained by biomass, which suggests either additional flow of C belowground per unit biomass at elevated CO_2, as has been shown at the plot-scale in BioCON (Adair *et al.*, 2009), or a change in the nature of rhizodeposits under elevated CO_2 that result in greater stimulation of SOM decomposition. The depressive effect of N addition on rhizosphere priming of decomposition was on par with the magnitude of the CO_2 effect: 29% on average when expressed per g soil C. However, we did not see a strong shift in plant allocation of resources in response to N, which suggest that the lower RPE with N addition may have resulted specifically from responses in exudation. Although it is understood that plants have some baseline rate of exudation (3-5% of total fixed C;

Pinton *et al.* 2001), there has been some evidence of plants' ability to reduce exudation rates in response to N fertilization (Phillips *et al.*, 2009; 2011).

We were surprised to find no evidence for a mediating effect of N addition on CO_2-induced increases in the RPE. The lack of interactive response in our study could relate to the relatively short-term nature of the experiment (one growing season). Although if this response were to hold, it would suggest that regardless of soil N status, elevated CO_2 may result in enhanced SOM decomposition from rhizosphere priming. However, the influence that N addition has on the CO_2-induced RPE response, however, seems to vary by system (Cardon, 1996; Phillips *et al.*, 2012). For example, a study of RPE effects to elevated CO_2 and N addition found an enhanced RPE under elevated CO_2 when the N level was high, and a lower RPE without added N (Cheng & Johnson, 1998). The diversity in observed responses to the interaction promote the idea that interactive effects of CO_2 and N could depend on other soil properties and nutrient status (Dijkstra *et al.*, 2013).

Species differences in RPE magnitude

The observed difference in rhizosphere priming of SOM decomposition between species merits further investigation. Although species differences in the RPE have previously been observed across functional groups (Fu & Cheng, 2002; Cheng, 2009), it was surprising to find such a difference in the magnitude of the RPE between two species of the same functional group. Our data suggest that characteristics other than total biomass –

or potential for C rhizosphere allocation – led to species differences. This could have occurred through differences in rhizodeposition amount or composition, or even the microbial communities the two species cultivated. Indeed, prior work has highlighted both species differences in root exudation composition and rates (Proctor & He, 2017), as well as the importance of exudates in controlling the RPE (Kuzyakov, 2002; Kuzyakov, 2010). Although our study included just two species, the observed variation in the magnitude of the RPE between them (as well as in other studies), suggests a broad range of potential C loss outcomes. It seems critical to develop a better understanding of plant species-level controls on RPE, as well as how they play out in a more diverse community, in order to predict widespread responses of C loss to global change.

In addition to global change and species treatment effects, we also expected the magnitude of the RPE would be influenced by three key plant and soil characteristics: 1) *total plant biomass*, where larger plants would have a larger RPE; 2) *soil C:N* as an indicator of N availability, where the RPE would be higher at higher soil C:N ratios; and 3) *activity of AMF*, where the RPE would be greater with more AMF activity. Below we address how our findings inform each of these expectations.

Plant biomass control of RPE

As discussed, and not surprisingly, plant biomass played a role in the observed RPE in response to elevated CO_2. Others have found greater RPEs with increased plant biomass, but specifically leaf biomass (Dijkstra *et al.*, 2006), likely due to its control on the

potential amount of fixed C available for allocation to the rhizosphere. Here, total biomass (used in the models) was highly positively related to shoot biomass (r=0.945, P<0.001), so a similar mechanism could be at play; however it was similarly positively related to root biomass (r=0.977, P<0.001), and more root biomass could also increase the possible sites where rhizosphere priming of SOM decomposition could occur. We are therefore unable to conclude if the RPE is greater with more plant biomass because there is more C fixed or more root surface area (or both).

Role of N availability (soil C:N) in RPE

Contrary to our expectation, we observed an overall smaller RPE at higher soil C:N (i.e. less SOM-derived C loss from rhizosphere priming when N was relatively less available). This contradicts the responses to the N addition treatment, and our hypothesis that the RPE would decrease at higher nutrient availability when plants are not N-limited, and likely not allocating extra C resources towards the rhizosphere. However, since soil N was determined through combustion of total N, as opposed to net N mineralization and nitrification, it may not accurately reflect available N. Given that, although soil C:N accounts for a portion of the variation in RPE response, we caution against deeply interpreting the effect observed here.

Role of AMF in the RPE

As we expected, we found more C lost from rhizosphere priming of SOM decomposition with greater AMF activity. There has been great interest in the potential for AMF to act

as decomposers, accelerating C loss under elevated CO_2, and potentially contributing to a positive C-climate feedback (Talbot *et al.*, 2008; Kowalchuk, 2012; Verbruggen *et al.*, 2013). Indeed, recent work has shown that AMF increase litter decomposition under elevated CO_2 and low N (Cheng *et al.*, 2012), and can result in greater soil C loss (Wurzburger & Brookshire, 2017). Our findings, while not a direct test of this question, do offer some evidence that AMF contribute to the RPE, and in this case potentially *Glomus* mycorrhizae in particular (given the positive relationship between RPE and the 16:1ω5c neutral lipid, which is most commonly found in *Glomus*: Graham et al. 1995). We also observed a trend towards even more rhizosphere priming of SOM decomposition with increased 16:1ω5c neutral lipid abundance under ambient N (Fig. S7), which follows prior suggestions that mycorrhizae may contribute to the RPE more when plants are nutrient limited (Cheng *et al.*, 2012).

Conclusions

Overall, we found substantial, but non-interacting, effects of elevated CO_2 and N addition on the amount of C released from rhizosphere priming of SOM decomposition, which collectively made up 0-39% of the total CO_2-C flux measured. Elevated CO_2 resulted in an average C loss of 34-39% from the RPE (on a per g soil and per g soil C basis, respectively), and N addition led to an average 29% loss of C from the RPE (on a per g soil C basis). We also found substantial differences in the amount of C lost from the RPE between two C3 grass species – either 340% or 460% depending on the metric of C flux used (on a per g soil and per g soil C basis, respectively). Additionally, we observed

positive relationships between the RPE and plant biomass and abundance of an AMF-associated neutral lipid (16:1ω5c). Collectively, our findings suggest that release of old, SOM-derived C could increase under elevated CO_2, even with increased N availability. If this effect holds for longer timeframes, or across different ecosystems, it would represent a meaningfully large additional input from soils that could result in a positive feedback, with implications for additional climate warming.

Acknowledgments

This work was supported by the following funding sources: the Cedar Creek Long Term Ecological Research Program (NSF DEB-9411972, DEB-0080302, DEB-0620652, and DEB-1234162), a NSF Doctoral Dissertation Improvement Grant (NSF 1501769), and the Inter-university Training in Continental-scale Ecology fellowship (NSF 1137336). CEK also received support from a NSF Graduate Research Fellowship (NSF 00039202). Additional thanks for the help of Geoff Harms with the whole-plant chamber design, Nolan O'Brien and Anika Bratt for assistance with fieldwork, and Dave Tilman and Nancy Johnson for helpful comments on manuscript drafts

Table 3.1. ANOVA table for plant characteristic responses to species, CO_2, N and microbial treatments (MBT=Microbial treatment). The response variables shoot C:N and shoot:root ratio were natural log-transformed and shoot %C, shoot %N, root %C, and root %N were arcsin-square root-transformed to meet model assumptions. Sample sizes vary slightly and are reported for different treatment combinations in Table S5. Marginal R^2 denotes the amount of variance explained by fixed effects alone, whereas Conditional R^2 is the amount of variance explained by both fixed and random effects.

Effect	**Total plant biomass** (g)	**Shoot biomass** (g)	**Shoot C** (%)	**Shoot N** (%)	**Shoot C:N**	**Root biomass** (g)	**Root C** (%)	**Root N** (%)	**Root C:N**	**Shoot:root ratio**
Species	****	****		**	***	****	***		****	****
CO_2	*	**				†		†		
N			†	****	****			**	****	
MBT										
Species*CO_2					†					
Species*N			†	****	****	*		*	**	**
Species*MBT							*		*	
CO_2*N	†					†				
CO_2*MBT	*					*				
N*MBT										
Species*CO_2*N								†		
Species*CO_2*MBT				†		*	*			
Species*N*MBT										†
CO_2*N*MBT	*					*				†
Species* CO_2*N*MBT			*							*
Marginal R^2	0.7687	0.7282	0.2040	0.6089	0.6353	0.7631	0.2552	0.2437	0.6184	0.5087
Conditional R^2	0.7821	0.7536	0.2040	0.6428	0.6885	0.7725	0.2552	0.2437	0.6184	0.5246

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 3.2. ANOVA table for soil and microbial characteristics responses to species, CO_2, N and microbial treatments (MBT=Microbial treatment). Microbial biomass C, N, and C:N were natural log-transformed to meet model assumptions. Sample sizes vary slightly and are reported for different treatment combinations in Table S5. Marginal R^2 denotes the amount of variance explained by fixed effects alone, whereas Conditional R^2 is the amount of variance explained by both fixed and random effects.

Effect	**Soil moisture** (%)	**Soil pH**	**Soil C** (%)	**Soil N** (%)	**Soil C:N**	**Microbial biomass C** (µg C/g soil)	**Microbial biomass N** (µg N/g soil)	**Microbial biomass C:N**
Species	****					**	****	
CO_2								
N	*		****	****	*	†	*	
MBT								
Species*CO_2								*
Species*N	*							
Species*MBT							†	†
CO_2*N				†				
CO_2*MBT								
N*MBT								
Species*CO_2*N							†	
Species*CO_2*MBT		†	†		*			
Species*N*MBT		†						
CO_2*N*MBT	**							
Species* CO_2*N*MBT								
Marginal R^2	0.4684	0.1596	0.2645	0.3452	0.1679	0.1808	0.3040	0.1766
Conditional R^2	0.4684	0.1596	0.2645	0.3452	0.1679	0.2170	0.3040	0.2055

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 3.3. ANOVA table for treatment effects on total microbial biomass, and abundance of fungal and bacterial biomarkers from PLFA and NLFA. MBT=Microbial treatment. Total microbial biomass and abundance of biomarkers (nmol/g soil) were square root- transformed to meet model assumptions when needed (a). The group the biomarker is an indicator for is in parentheses: arbuscular mycorrhizal fungi (AMF), general saprotrophic fungi (GF), Gram positive bacteria (Gram +), and Gram negative bacteria (Gram-).

	Total microbial biomass	**Lipid Abundance**							
Effect		**16:1ω5c PLFA (AMF)**	**16:1ω 5c NLFA (AMF)**	**18:1ω9c PLFA (AMF/GF)[a]**	**18:1ω9c NLFA (AMF/GF)[a]**	**18:2ω6,9c PLFA (GF)[a]**	**18:2ω6,9c NLFA (GF)[a]**	**15:0 iso PLFA (Gram +)[a]**	**16:1ω7c PLFA (Gram -)[a]**
Species		****				*		*	†
CO_2									
N	†							*	
MBT	†	**	*	**	†			*	**
Species*CO_2			*		*				
Species*N									
Species* MBT	*	**		†				*	*
CO_2*N		*	†			*		†	
CO_2* MBT									
N* MBT									
Species*CO_2*N		†			†				
Species*CO_2* MBT		*							
Species*N* MBT									
CO_2*N* MBT									
Species* CO_2*N* MBT									
Marginal R^2	0.21	0.36	0.22	0.24	0.18	0.22	0.09	0.31	0.23
Conditional R^2	0.21	0.36	0.22	0.24	0.18	0.22	0.09	0.32	0.27

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 3.4. ANOVA table for CO_2 flux and priming responses to species, CO_2, N and microbial treatments (MBT=Microbial treatment). Response variables proportion of flux from SOM was arcsin square root-transformed and SOM-derived C flux (mgC/hr/gsoilC) was natural log-transformed to meet model assumptions. Sample sizes vary slightly and are reported for different treatment combinations in Table S5. Marginal R^2 denotes the amount of variance explained by fixed effects alone, whereas Conditional R^2 is the amount of variance explained by both fixed and random effects.

Effect	Total CO_2 flux	Total CO_2 flux	SOM Proportion of total flux	SOM-derived C flux	SOM-derived C flux	Primed C	Primed C	Priming Proportion of total flux	Priming Proportion of total flux
	(mg C/hr/g soil)	(mg C/hr/g soil C)		(mg C/hr/g soil)	(mg C/hr/g soil C)	(mg C/hr /g soil)	(mg C/hr/g soil C)	(with mg C/hr/g soil)	(with mg C/hr/g soil C)
Species	****	****	****	****	****	***	****	****	****
CO_2	*	*	†	*	*	*	*	*	
N		*	†	*	****		**		†
MBT									*
Species*CO_2								**	*
Species*N									
Species*MBT								†	†
CO_2*N									
CO_2*MBT									
N*MBT						†			
Species*CO_2*N									
Species*CO_2*MBT									
Species*N*MBT									
CO_2*N*MBT				†		†			
Species* CO_2*N*MBT									
Marginal R^2	0.7591	0.6508	0.5961	0.6916	0.5618	0.6926	0.4935	0.5370	0.4200
Conditional R^2	0.7892	0.6940	0.5961	0.7047	0.5618	0.7056	0.5010	0.5370	0.4200

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Table 3.5. ANOVA table for covariate rhizosphere priming effect models. MBT = Microbial treatment. Sample sizes vary slightly and are reported for different treatment combinations in Table S5. Marginal R^2 denotes the amount of variance explained by fixed effects alone, whereas Conditional R^2 is the amount of variance explained by both fixed and random effects.

Effect	**Primed C** (mg C/hr /g soil)	**Primed C** (mg C/hr/g soil C)
Species	****	**
CO_2	*	†
N		***
MBT		†
Soil C:N		****
Total plant biomass		†
16:1ω5c neutral lipid	†	**
Species*CO_2		
Species*N		
Species*MBT		
CO_2*N		
CO_2*MBT		
N* MBT		
Species*CO_2*N		
Species*CO_2*MBT		
Species*N*MBT		
CO_2*N*MBT	†	
Species* CO_2*N*MBT		
Marginal R^2	0.6961	0.6114
Conditional R^2	0.7033	0.6263

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

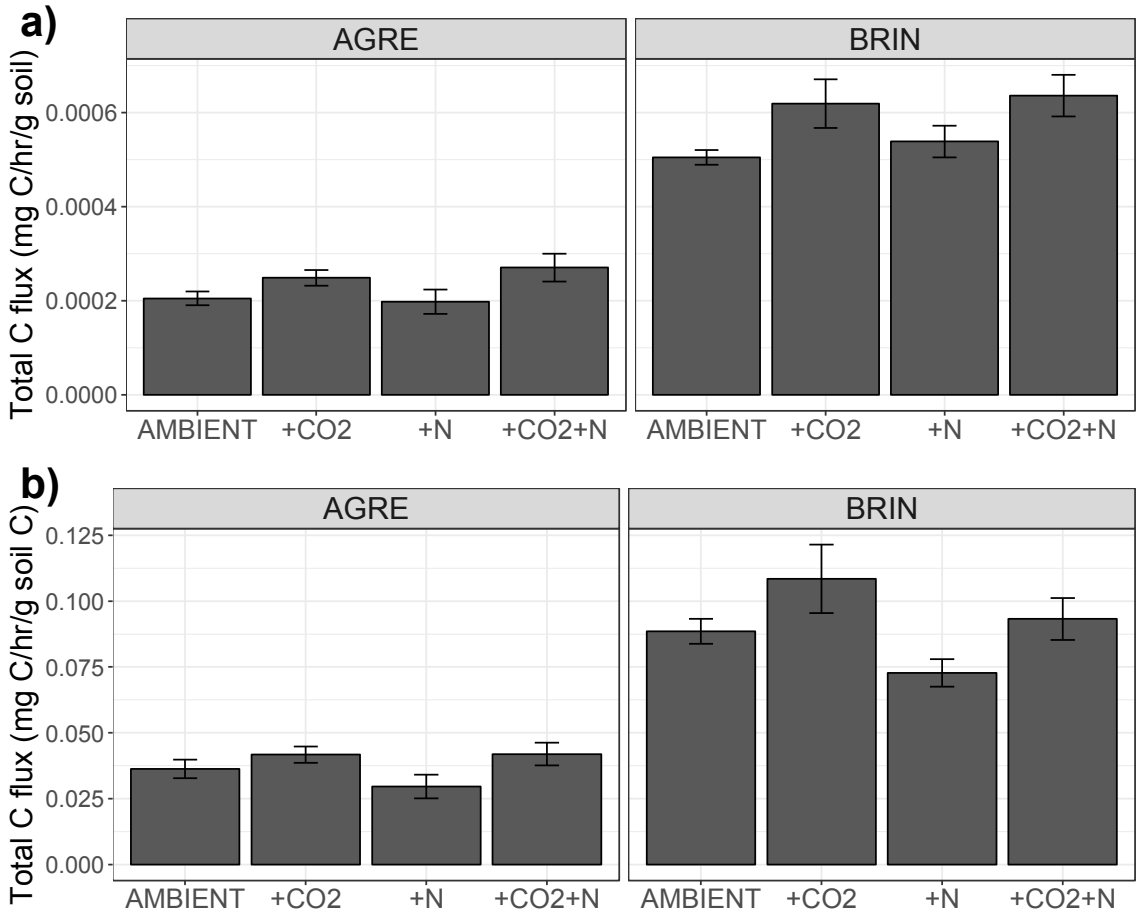


Figure 3.1. Total CO2-C flux across plant species, CO_2, and N treatments, expressed as **a)** mg C / hr / g soil and **b)** mg C / hr / g soil C. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*. Sample sizes vary slightly and are reported for different treatment combinations in Table S5.

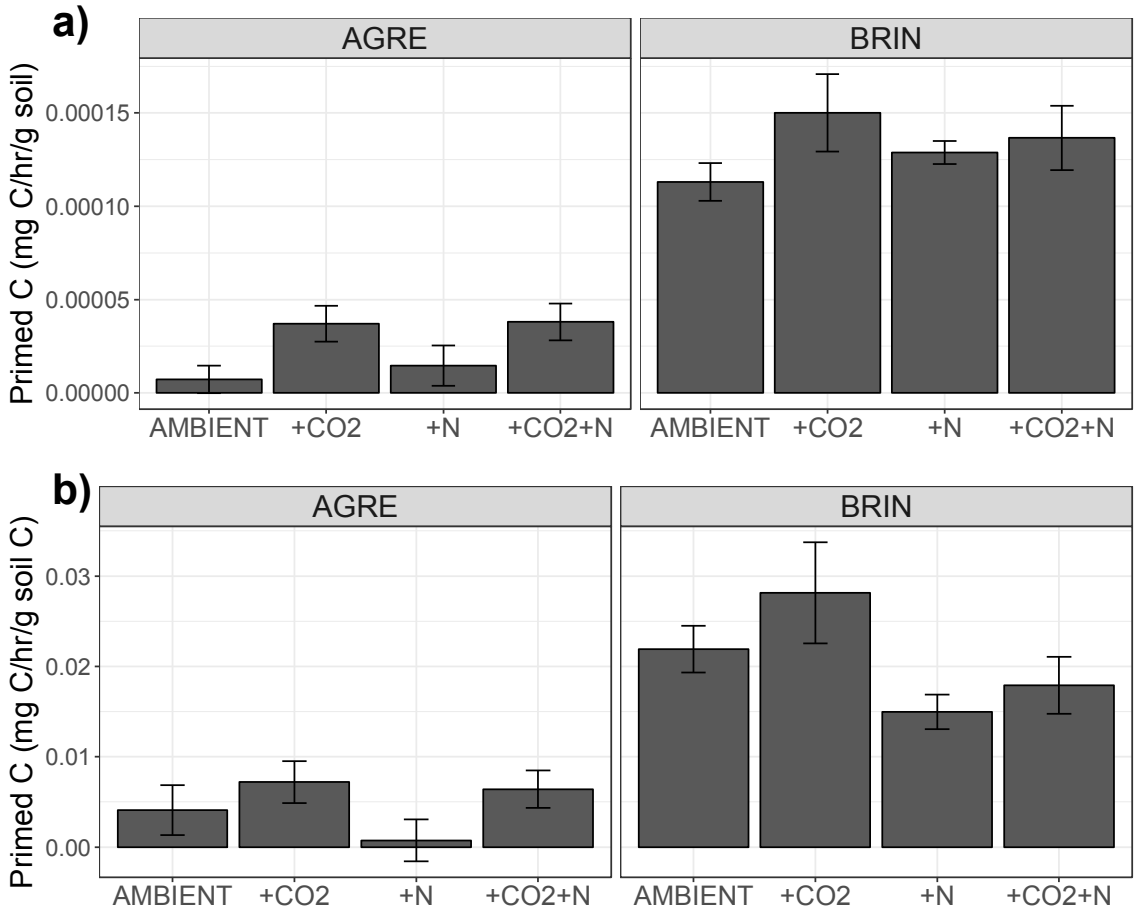


Figure 3.2. The amount of SOM-C from rhizosphere priming, across plant species, CO_2, and N treatments, expressed as **a)** mg C / hr / g soil and **b)** mg C / hr / g soil C. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*. Sample sizes vary slightly and are reported for different treatment combinations in Table S5.

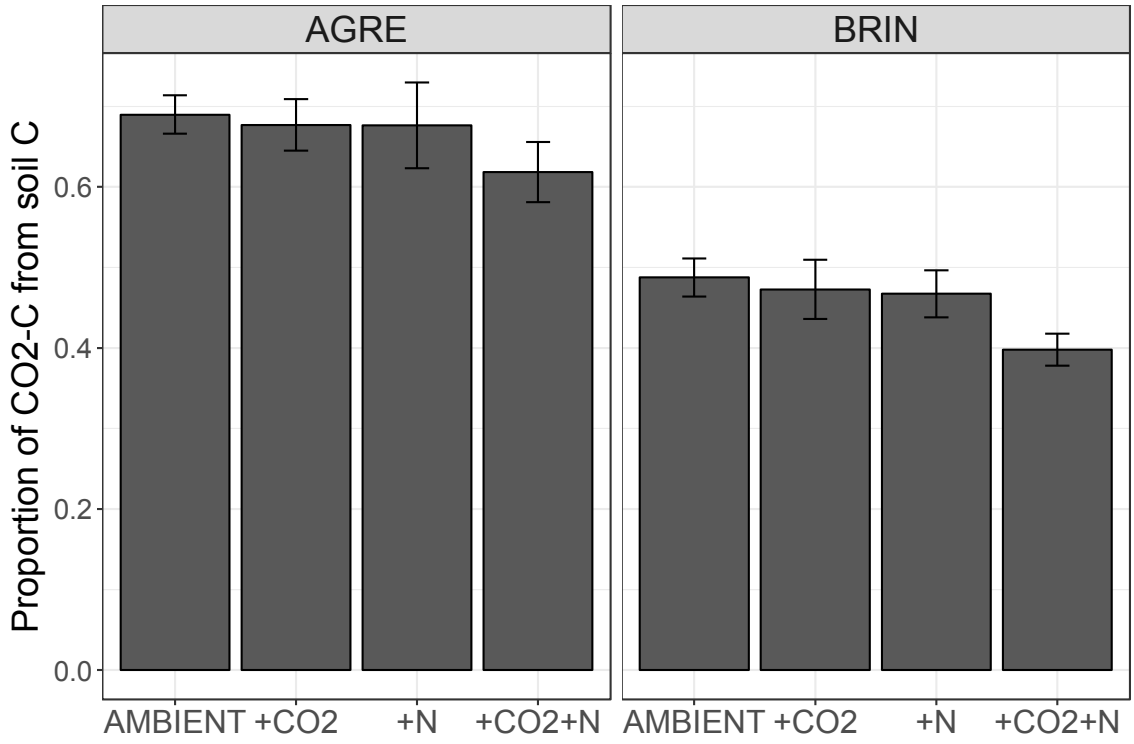


Figure 3.3. The proportion (0-1) of total CO_2-C flux from soil C across plant species, CO_2, and N treatments. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*.

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APPENDIX 1 – CHAPTER 1 SUPPLEMENTAL MATERIAL

Table S1.1. ANOVA table for aggregate fraction %C across time. %C in aggregate fractions was logit-transformed to meet model assumptions. We did not include GWC in models, as there is not a clear mechanistic reason for inclusion. GWC was also not correlated with %C in most fractions (although the medium macroaggregates were negatively correlated with GWC, r=-0.44, P<0.001).

Effect	%C in Large macroaggregates (>2mm)		%C in Medium macroaggregates (1-2mm)		%C in Small macroaggregates (1mm - 250µm)		%C in Microaggregates (>250µm)	
	2012	2013	2012	2013	2012	2013	2012	2013
CO_2	†		†		†			
Nitrogen	†					†		
Bag	†		**	†		**		†
CO_2 * Nitrogen								†
CO_2 * Bag			***			*		
Nitrogen * Bag								
CO_2 * Nitrogen * Bag					*			
Marginal R^2	0.13	0.05	0.27	0.10	0.12	0.14	0.03	0.10
Conditional R^2	0.13	0.05	0.38	0.24	0.17	0.35	0.03	0.11

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001

Table S1.2. ANOVA table for plot-level environmental conditions. Data is from BioCON plots and was collected over the growing seasons of 2012 and 2013. Soil moisture was determined from TDR measurements over the growing season, total root biomass was determined at the height of the growing season, and annual root production was measured using an in-growth core in the plot. We used mixed effects models with CO_2, N, and Year as fixed effects and ring nested in CO_2 as a random effect. To meet model assumptions total root biomass and annual root production was log-transformed.

	Soil Moisture (%)	**Total Root Biomass (g/m^2)**	**Annual Root Production (g/m^2)**
CO_2			
Nitrogen	*		
Year		***	
CO_2 * Nitrogen			
CO_2 * Year	†		
Nitrogen * Year			
CO_2 * Nitrogen * Year			
Marginal R^2	0.01	0.32	0.05
Conditional R^2	0.04	0.32	0.10

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

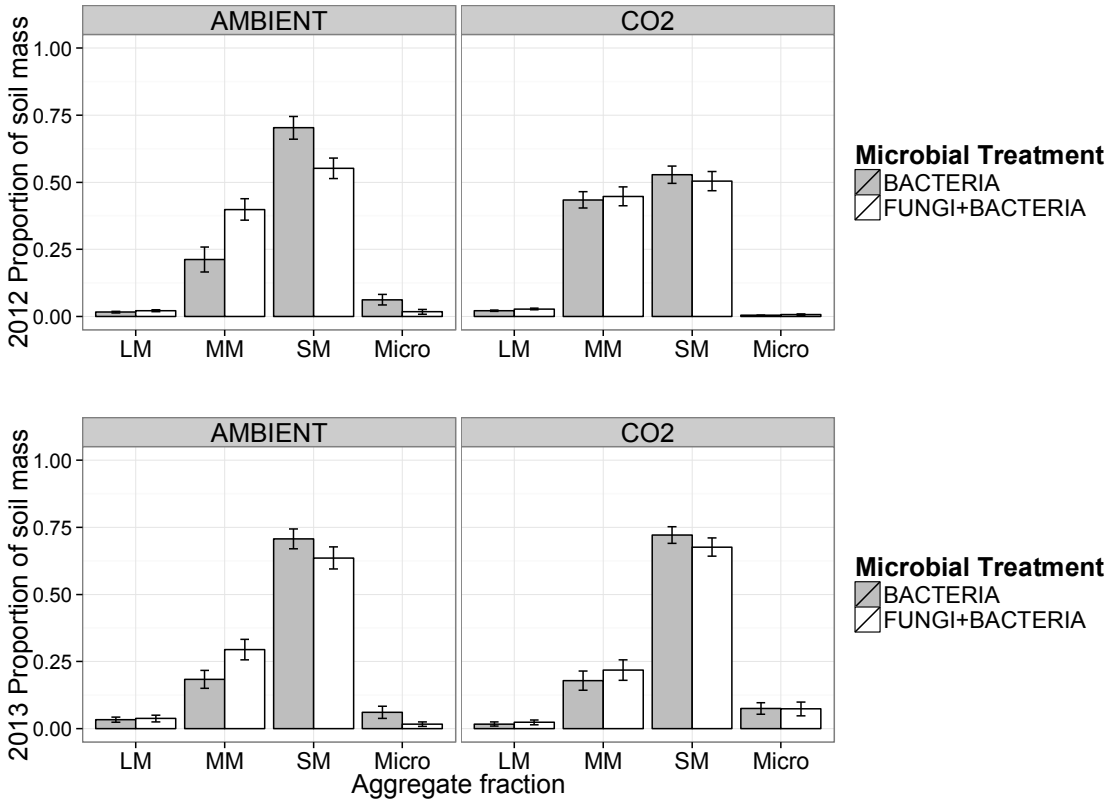

Figure S1.1. CO_2 and in-growth bag effects on aggregate fractions over time.

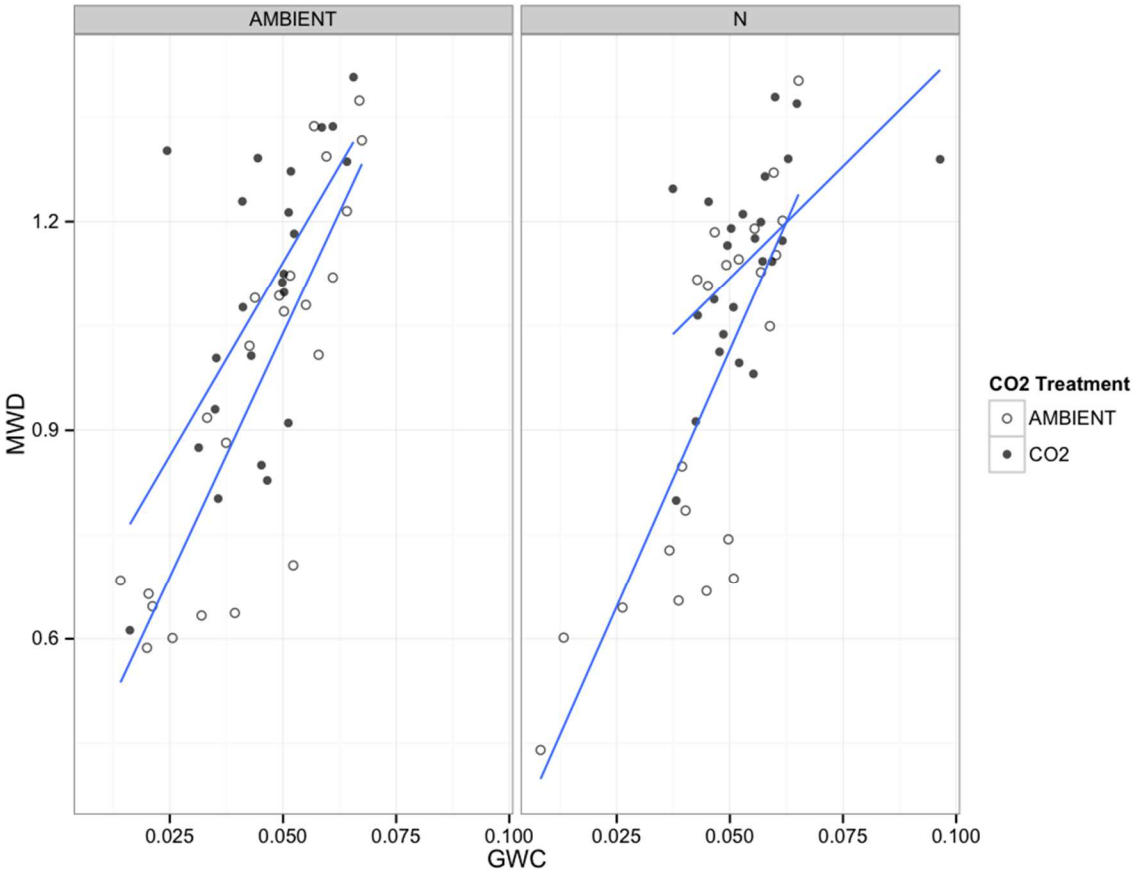

Figure S1.2. 2012 aggregate mean-weighted diameter (MWD) by CO_2 and N treatments and gravimetric water content (GWC) at the time soils were sieved.

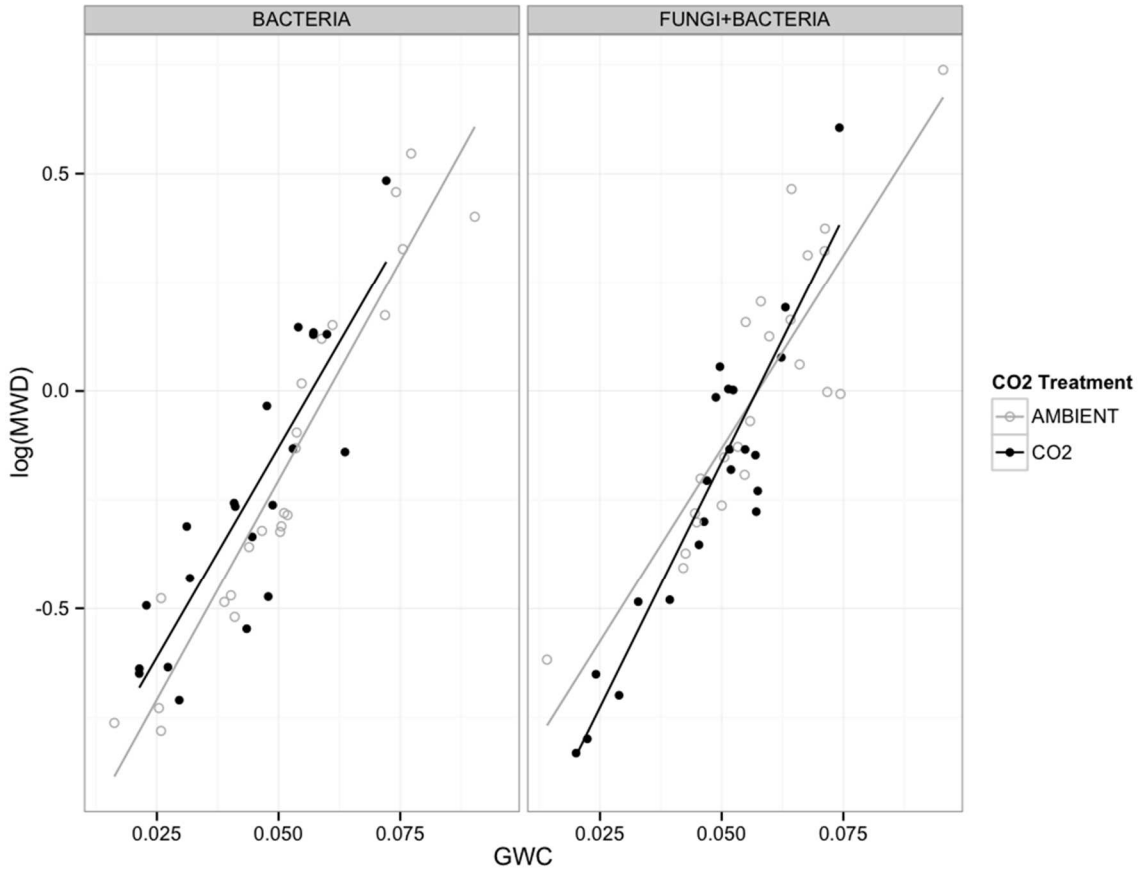

Figure S1.3. 2013 aggregate mean-weighted diameter (MWD) by CO_2 and in-growth bag type and gravimetric water content (GWC). MWD is log-transformed to meet model assumptions.

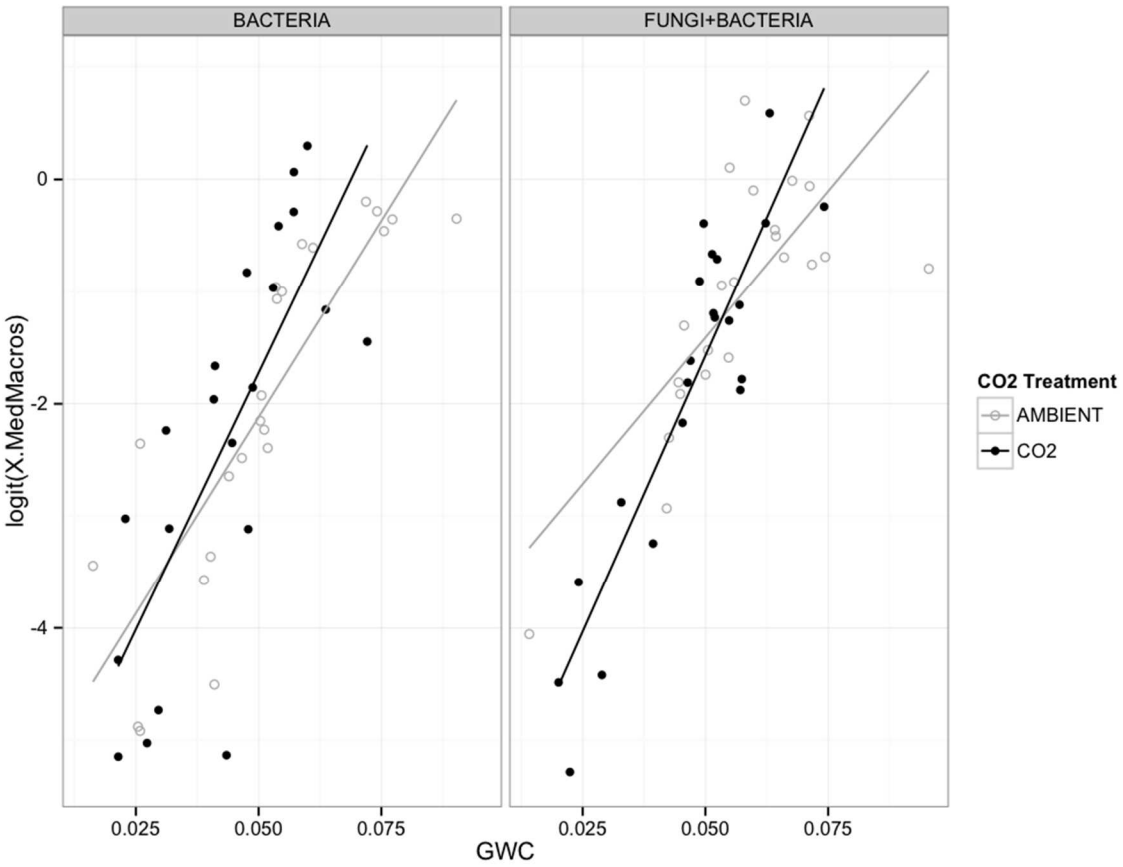

Figure S1.4. 2013 proportion of soil in medium macroaggregates (1-2mm) by CO_2 and in-growth bag type, and gravimetric water content (GWC). Medium macroaggregate fraction is logit-transformed to meet model assumptions.

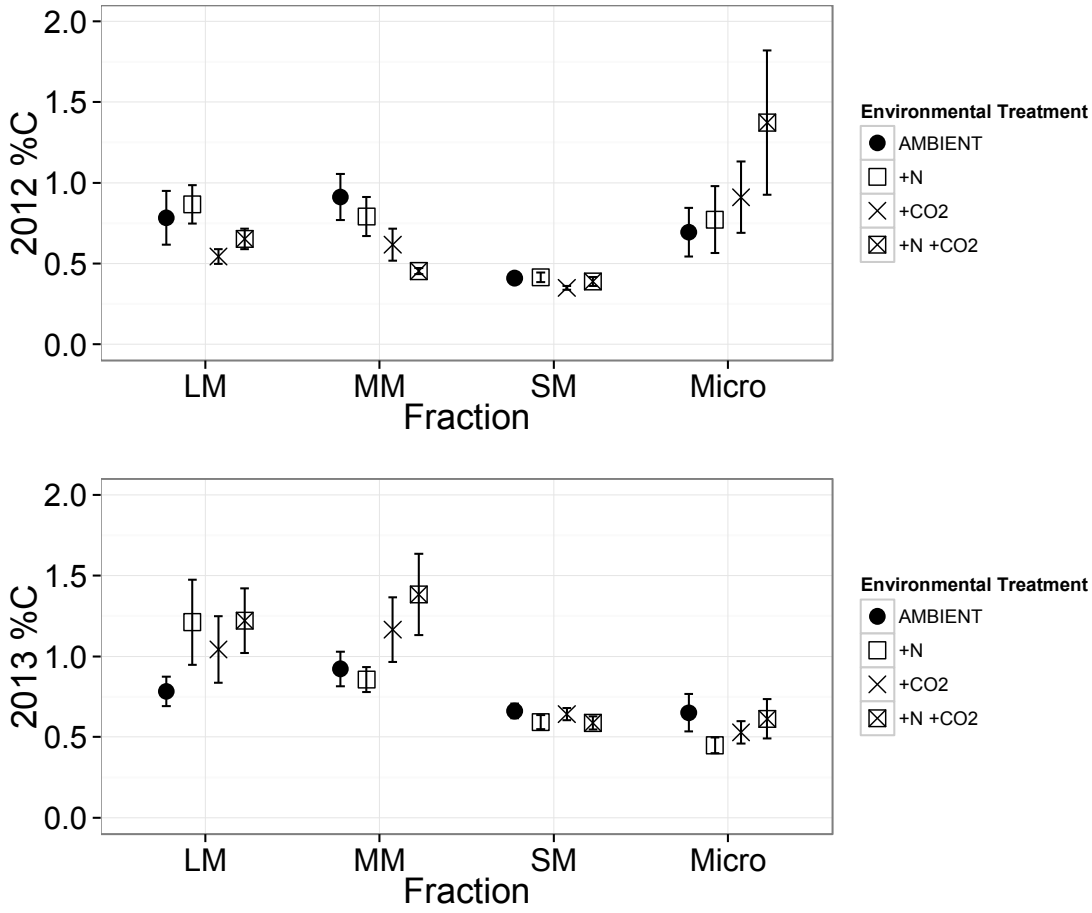

Figure S1.5. CO_2 effects on %C in aggregate fractions over time.

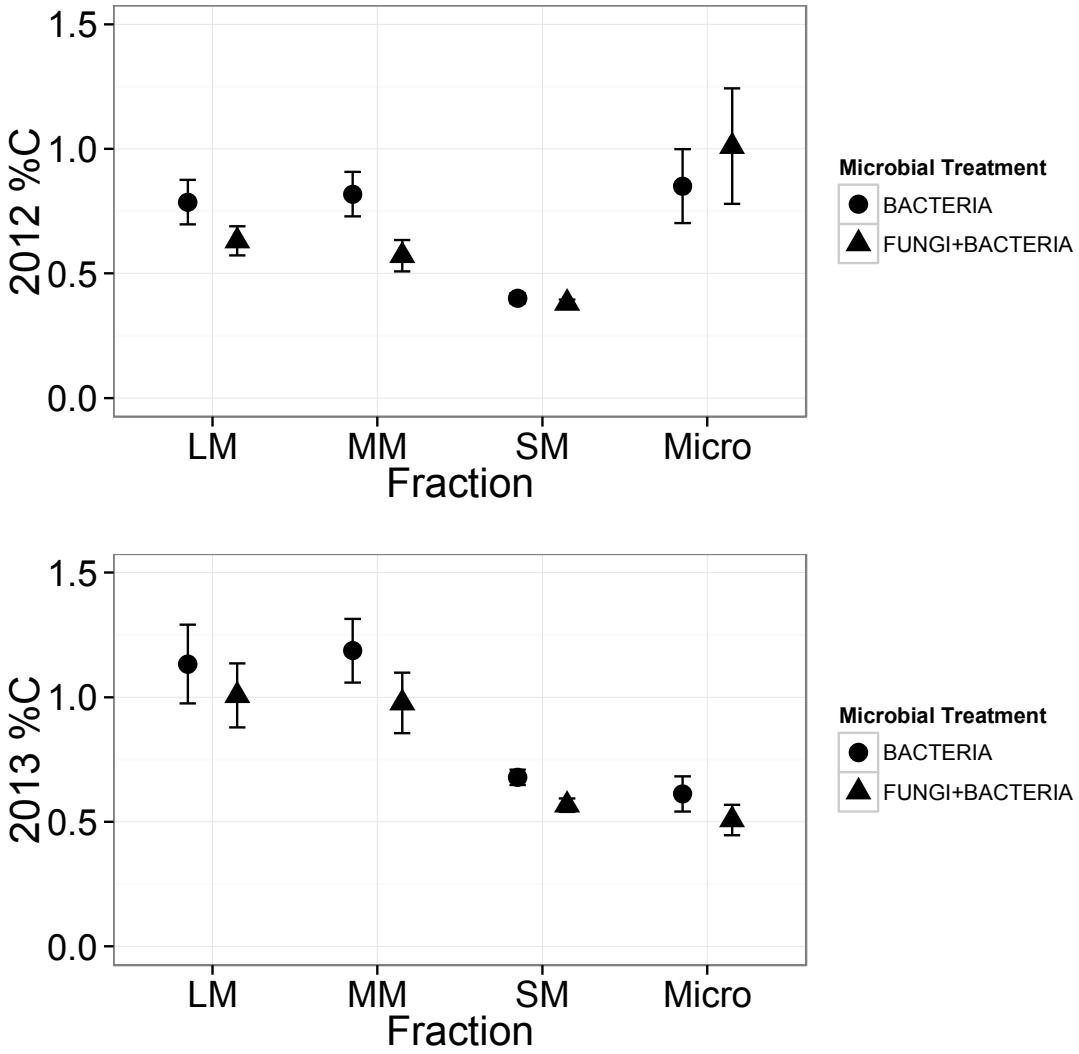

Figure S1.6. In-growth bag effects on %C in aggregate fractions over time.

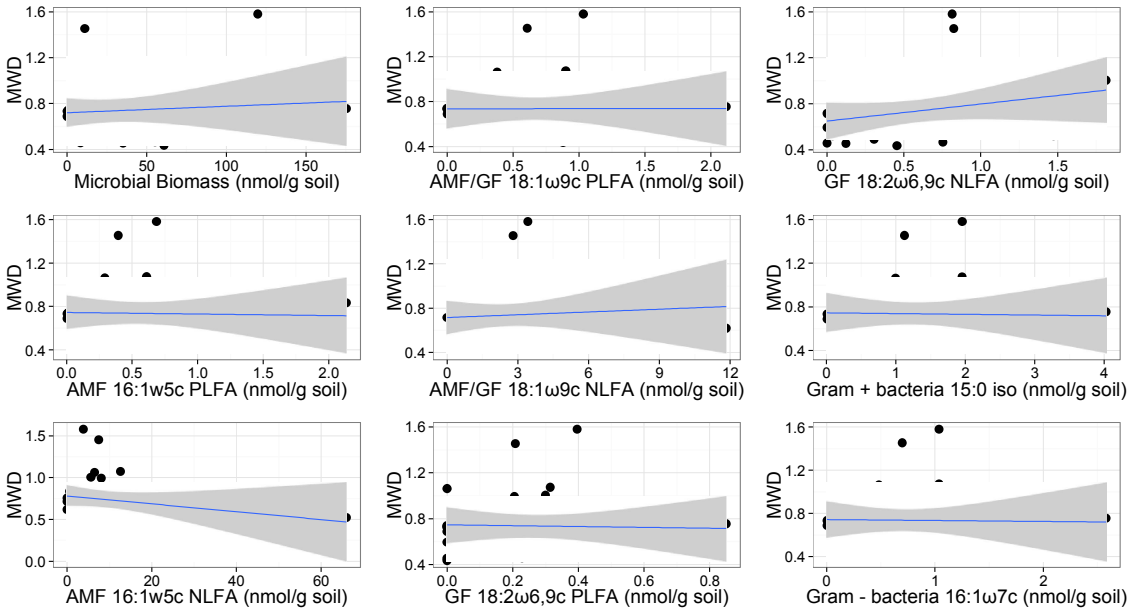

Figure S1.7. Relationships between microbial biomass and individual fungal and bacterial biomarker abundance and aggregate mean-weight diameter (MWD).

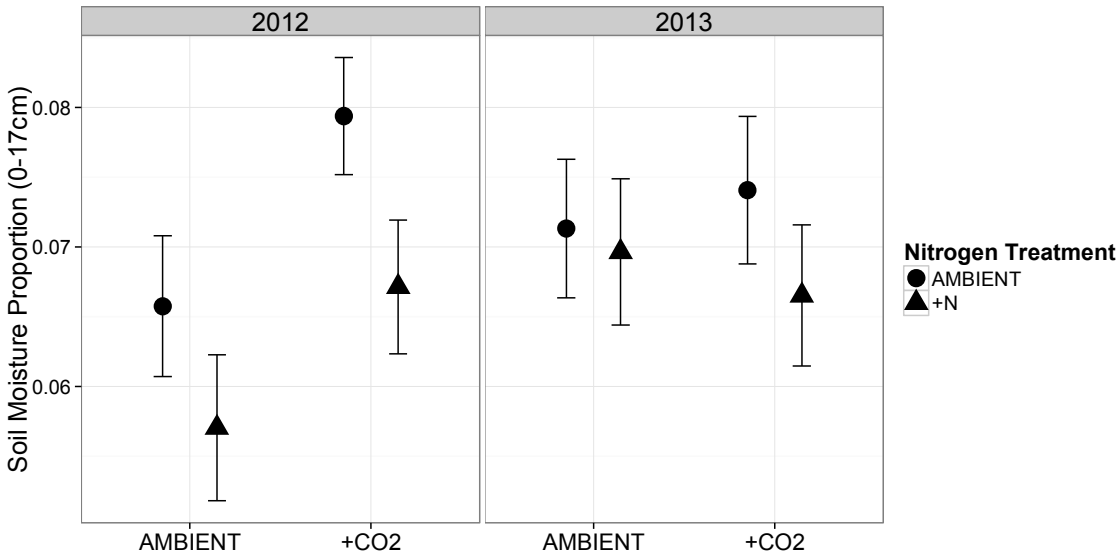

Figure S1.8. Plot-level soil moisture by CO_2 and N treatments across years. Soil moisture was measured using a TDR probe multiple times throughout each growing season. Annual averages reported here were from measurements taken between April and October (when in-growth bags were harvested).

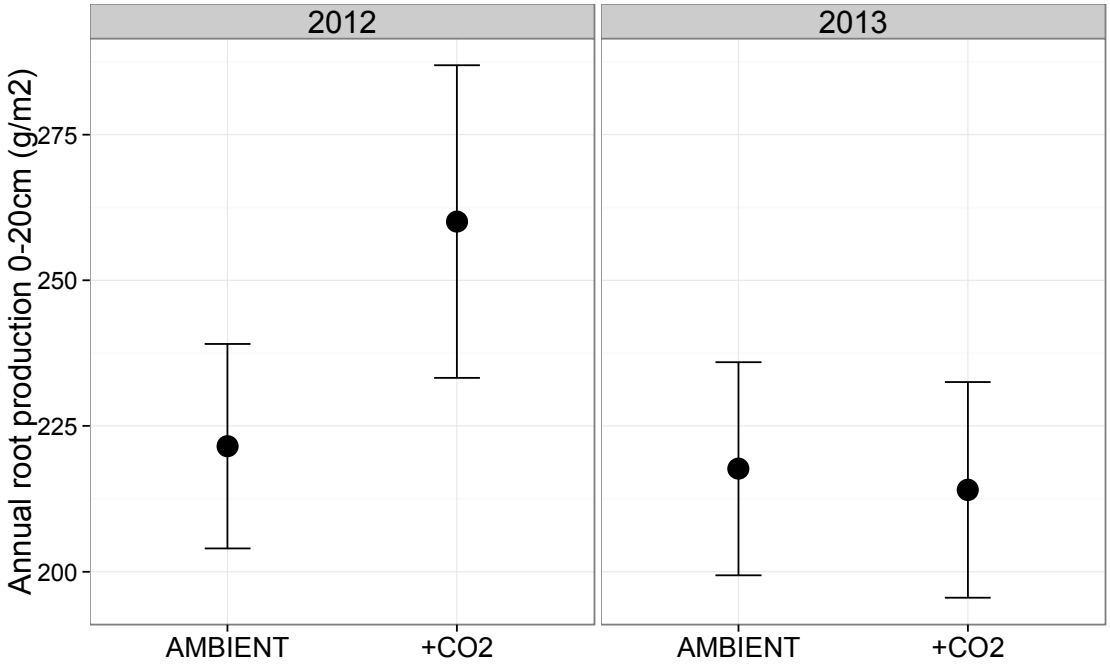

Figure S1.9. Root productionby CO_2 and N treatments across years. Annual root production was assessed from in-growth cores in the plots at BioCON.

APPENDIX 2 – CHAPTER 2 SUPPLEMENTAL MATERIAL

Table S2.1. Correlation coefficients for all covariates from averaged site-level values from ambient only plots. Units for characteristics are as follows: soil C and N are in %, soil C:N is unitless, fine root biomass is in g/m2, soil moisture is in %, soil pH is unitless, microbial C and N (MB C and MBN, respectively) are in µg C/g soil and microbial biomass C:N (MB C:N) is unitless. Bolded coefficients are statistically significant (*** P <0.001, ** 0.001 – 0.01, * 0.01 – 0.05).

	Soil N	Soil C:N	Fine root biomass	Soil moisture	Soil pH	MBC	MB N	MB C:N
Soil C	**0.95*** **	**0.73*** **	**- 0.37*** **	**0.70*** **	**- 0.85*** **	**0.72*** **	**0.72*** **	**-0.47 *** **
Soil N		**0.51*** **	**- 0.29** **	**0.75*** **	**- 0.93*** **	**0.72*** **	**0.74*** **	**-0.58*** **
Soil C:N			**- 0.49*** **	**0.28** **	**- 0.43*** **	**0.41*** **	**0.38*** **	0.02
Fine root biomass				**0.30** **	**0.46*** **	**0.25***	**0.28** **	**-0.42*** **
Soil moisture					**- 0.60*** **	**0.93*** **	**0.96*** **	**-0.85*** **
Soil pH						**-0.59*** **	**-0.59*** **	**0.37*** **
MB C							**0.99*** **	**-0.69*** **
MB N								**-0.78*** **

Table S2.2. ANOVA Table for decay rates, pool sizes, and cumulative microbial respiration per gram soil C for simple, hypothesis-testing model (Response ~ Site*N). Response variables were log-transformed, as noted, when necessary to meet model assumptions.

Effect	Log(k_f)	Log(k_s)	C_f	Log(C_s)	Log(Respiration)
Site	****	****	**	****	***
N					
Site x N	NA	NA	†	NA	NA
Adjusted R^2	0.3342	0.3261	0.2065	0.8086	0.3505

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, NA = non-significant interaction term removed from model

Table S2.3. ANOVA Table for decay rates, pool sizes, and cumulative microbial respiration per gram soil C from full model. Response variables were log-transformed to meet model assumptions.

Effect	Log(k_f)	Log(k_s)	Log(C_f)	Log(C_s)	Log(Respiration)
Site	*	***			*
N					
Soil %C	**	**	*	****	
Soil C:N					
Microbial biomass C		*			*
Fine root biomass		****			****
Soil %C x N	NA	NA	NA	NA	NA
Soil C:N x N	NA	NA	NA	NA	NA
Microbial biomass C x N	NA	NA	NA	NA	NA
Fine root biomass x N	NA	NA	NA	NA	NA
Site x N	NA	NA	NA	NA	NA
Adjusted R^2	0.3342	0.3051	0.1468	0.8086	0.3505

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model

Table S2.4. ANOVA Table for fine root chemistry (%C, %N, C:N, lignin:N, % soluble cell contents, % hemicellulose and bound proteins, % cellulose, % lignin). Two-way ANOVAs were performed for each metric of root chemistry and its response to Site and N Treatment.

Characteristic	***Site***	***Treatment***	***Site*Treatment***	***Adjusted R^2***
Fine root %C	**			0.1627
Fine root %N	***	***	***	0.5515
Logged fine root C:N	***	***	*	0.3587
Logged fine root lignin:N		***		0.0953
Fine root % soluble cell contents	***	*		0.3521
Logged fine root % hemicellulose and bound proteins	***			0.506
Fine root % cellulose				0.01503
Fine root % lignin	*			0.08229

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

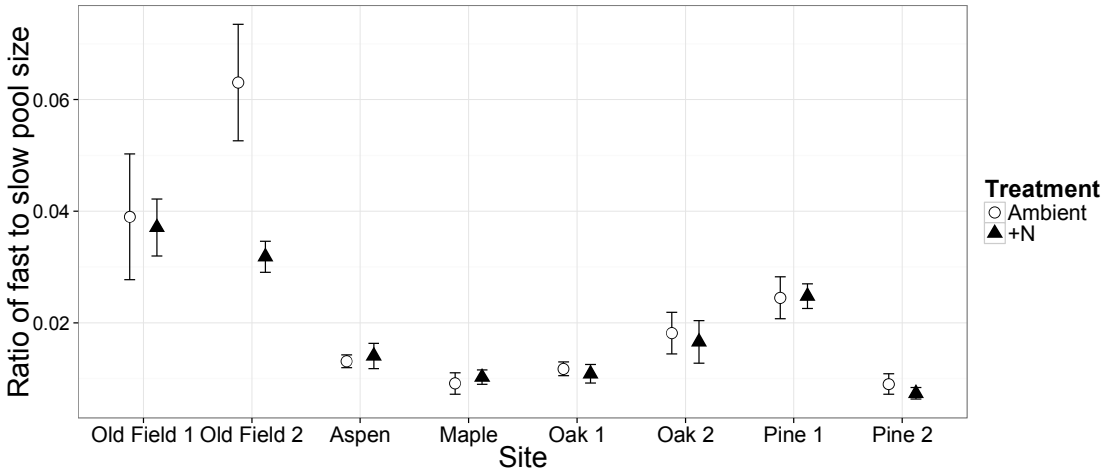

Figure S2.1. The relative amount of C in the fast pool compared to the slow pool (C_f:C_s) by site and N treatment.

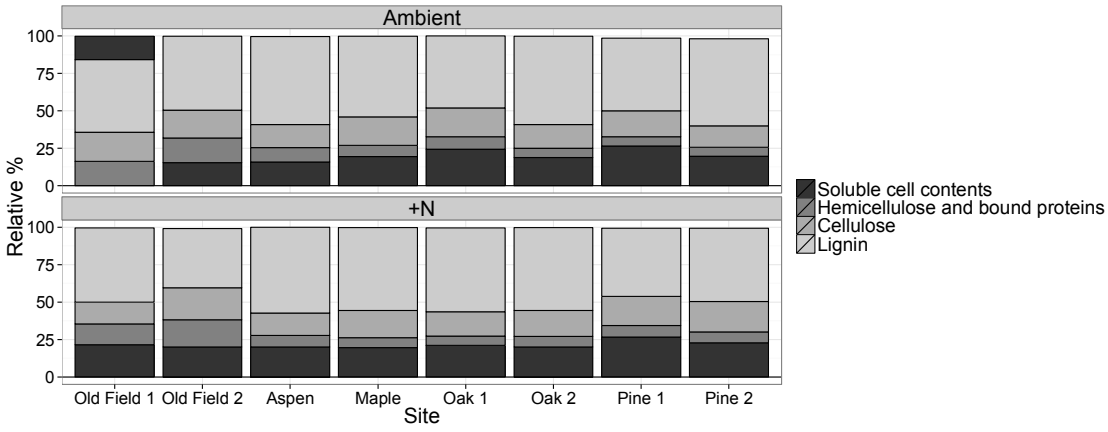

Figure S2.2. Fine root tissue chemistry in ambient and N addition plots.

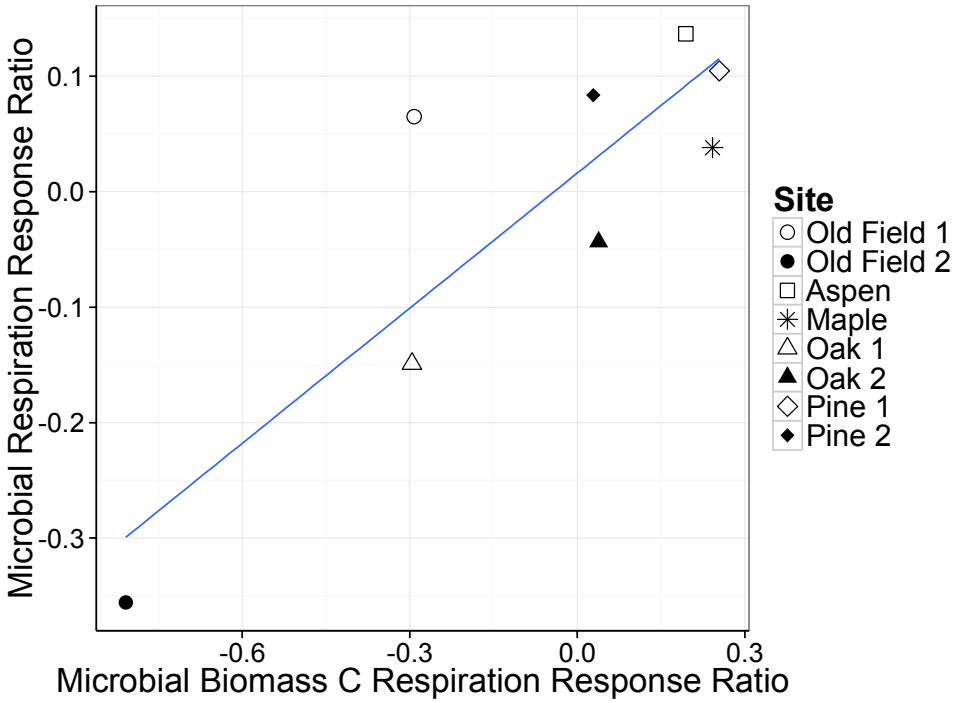

Figure S2.3. Comparison of response ratios of microbial respiration per gram soil C and microbial biomass C. Site-level response ratios are calculated from the six ambient and six +N plots (with the exception of Old Field 1 where microbial biomass C n=5). The relationship can be described by $y = 0.3898*x + 0.0160$ ($R^2 = 0.7052$, $P=0.0056$).

APPENDIX 3 – CHAPTER 3 SUPPLEMENTAL MATERIAL

Table S3.1. Average plant, soil, and CO_2 $\delta^{13}C$ values across species, CO_2, N, and microbial treatments. For the microbial treatment, *REST*=the restricted microbial treatment (<53µm) and *INC*=the inclusive microbial treatment (>53µm). Root $\delta^{13}C$ was used as the plant end member and the unplanted CO_2 $\delta^{13}C$ was used as the soil end member. The unplanted CO_2 $\delta^{13}C$ was not significantly different between treatments (P>0.1), so was averaged for mixing model calculations to -15.25 (0.29). Means are reported with one standard error. Sample sizes vary slightly and are reported for different treatment combinations in Table S5.

	Agropyron repens								***Bromus inermis***							
	AMBIENT		**+ CO_2**		**+N**		**+CO_2 +N**		**AMBIENT**		**+ CO_2**		**+N**		**+CO_2 +N**	
	REST	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*
CO_2 $\delta^{13}C$	-19.48	-18.75	-21.05	-22.32	-18.63	-19.58	-23.97	-22.38	-21.16	-21.43	-25.56	-27.39	-21.56	-21.36	-27.68	-27.57
	(0.42)	(0.29)	(1.01)	(0.99)	(1.82)	(1.82)	(1.76)	(0.94)	(0.30)	(0.36)	(1.20)	(0.56)	(0.42)	(0.49)	(0.89)	(0.59)
Shoot $\delta^{13}C$	-28.48	-29.35	-33.80	-34.51	-27.74	-29.40	-32.52	-33.44	-28.60	-28.86	-38.31	-37.33	-28.69	-28.48	-37.75	-37.74
	(0.20)	(1.42)	(1.50)	(0.66)	(0.49)	(1.39)	(2.63)	(0.74)	(0.30)	(0.18)	(0.79)	(1.01)	(0.30)	(0.23)	(0.51)	(0.61)
Root $\delta^{13}C$	-28.09	-27.33	-34.63	-34.32	-27.20	-27.35	-35.76	-33.76	-26.85	-27.35	-36.65	-36.41	-27.01	-26.93	-35.72	-35.87
	(0.13)	(0.37)	(0.95)	(0.71)	(0.69)	(0.24)	(0.15)	(0.74)	(0.33)	(0.22)	(0.82)	(0.71)	(0.27)	(0.16)	(0.55)	(0.77)
Soil $\delta^{13}C$	-21.78	-21.06	-21.59	-22.05	-22.15	-21.34	-21.34	-21.91	-21.59	-21.91	-22.13	-21.78	-21.50	-21.53	-21.82	-21.93
	(0.20)	(0.21)	(0.19)	(0.21)	(0.21)	(0.25)	(0.24)	(0.27)	(0.17)	(0.24)	(0.17)	(0.24)	(0.10)	(0.11)	(0.23)	(0.54)
Unplanted CO_2 $\delta^{13}C$	*No plant species*								-14.50	-15.12			- 15.85	-15.53		
									(1.01)	(0.36)			(0.25)	(0.33)		

Table S3.2. Correlation coefficients for all covariates across all species, CO_2, N and microbial treatment combinations (MBT=Microbial treatment). Bolded coefficients are statistically significant. Sample sizes vary slightly and are reported for different treatment combinations in Table S5.

	Soil N	Soil C:N	Soil moisture	Soil pH	Total plant biomass	Shoot biomass	Shoot C	Shoot N	Shoot C:N	Root biomass	Root C	Root N	Root C:N	Shoot: root	MB C	MB N	MB C:N
Soil C	**0.928** ***	**0.391** ***	**-0.351** **	0.003	0.078	0.095	0.031	**0.434** ***	**-0.376** **	0.062	-0.088	**0.266** *	**-0.389** ***	-0.097	0	0.181	-0.037
Soil N	*****	0.023	**-0.291** **	-0.036	0.048	0.081	-0.045	**0.51** ***	**-0.447** ****	0.024	-0.07	**0.351** **	**-0.455** ***	-0.039	0.026	0.216 †	-0.044
Soil C:N		*****	-0.207 †	0.089	0.068	0.031	0.199 †	-0.058	0.061	0.088	-0.053	-0.128	0.064	-0.162	-0.066	-0.07	0.027
Soil moisture			*****	-0.196 †	**-0.566** ***	**-0.579** ***	0.012	-0.109	0.077	**-0.523** ***	**0.338** **	-0.131	**0.527** ***	**0.232** *	-0.108	-0.164	0.111
Soil pH				*****	0.107	0.13	0.052	-0.084	0.025	0.086	-0.104	-0.175	0.071	0.022	-0.025	-0.077	0.181
Total plant biomass					*****	**0.945** ***	-0.023	**-0.245** *	**0.308** **	**0.977** ***	**-0.489** ***	-0.067	**-0.485** ***	**-0.543** ***	0.139	**0.315** **	**-0.232** *
Shoot biomass						*****	-0.023	-0.192 †	0.29	**0.854** ***	**-0.419** ***	0.031	**-0.512** ***	**-0.378** **	0.141	**0.337** **	**-0.249** *
Shoot C							*****	-0.127	**0.295** **	-0.022	-0.057	-0.133	0.09	-0.071	0.013	-0.065	0.078
Shoot N								*****	**-0.93** ***	**-0.266** *	0.06	**0.371** ***	**-0.333** **	0.035	0.055	0.11	0.024
Shoot C:N									*****	**0.301** **	-0.091	**-0.307** **	**0.226** *	-0.074	-0.041	-0.075	-0.066
Root biomass										*****	**-0.506** ***	-0.127	**-0.44** ***	**-0.619** ***	0.136	**0.286** *	-0.199 †
Root C											*****	**0.619** ***	**0.468** ***	**0.281** *	-0.174	**-0.313** **	0.052
Root N												*****	**-0.394** ***	0.084	-0.031	-0.007	-0.14
Root C:N													*****	**0.229** *	-0.181	**-0.383** **	**0.24** *
Shoot: root														*****	-0.092	**-0.235** *	**0.236** *
MB C															*****	**0.666** ***	0.048
MB N																*****	**-0.567** ***

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001

Table S3.3. Average plant biomass and tissues chemistry across species, CO_2, N and microbial treatments. For the microbial treatment, *REST*=the restricted microbial treatment (<53μm) and *INC*=the inclusive microbial treatment (>53μm). Means are reported with one standard error. Sample sizes vary slightly and are reported for different treatment combinations in Table S5.

	Agropyron repens								***Bromus inermis***							
	AMBIENT		**+ CO_2**		**+N**		**+CO_2 +N**		**AMBIENT**		**+ CO_2**		**+N**		**+CO_2 +N**	
	REST	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*
Total biomass (g)	0.340 (0.028)	0.413 (0.075)	0.318 (0.054)	0.649 (0.255)	0.396 (0.206)	0.410 (0.092)	0.664 (0.224)	0.569 (0.139)	1.570 (0.146)	1.332 (0.097)	1.321 (0.206)	1.817 (0.245)	1.196 (0.092)	1.223 (0.107)	1.574 (0.189)	1.675 (0.066)
Shoot biomass (g)	0.194 (0.012)	0.234 (0.046)	0.208 (0.030)	0.332 (0.069)	0.180 (0.101)	0.222 (0.058)	0.340 (0.105)	0.347 (0.061)	0.593 (0.037)	0.589 (0.032)	0.605 (0.116)	0.776 (0.116)	0.595 (0.038)	0.590 (0.053)	0.792 (0.061)	0.750 (0.057)
Shoot %C	41.731 (0.379)	42.769 (0.865)	43.452 (0.410)	42.525 (0.529)	43.094 (0.621)	42.331 (1.018)	42.617 (0.677)	42.815 (0.676)	43.220 (0.348)	42.828 (0.894)	42.920 (0.346)	43.228 (0.607)	41.498 (0.946)	42.303 (0.540)	42.878 (0.364)	40.163 (1.622)
Shoot %N	3.792 (0.208)	3.977 (0.270)	3.763 (0.351)	4.019 (0.379)	4.534 (0.129)	4.145 (0.218)	4.239 (0.286)	4.552 (0.131)	2.927 (0.143)	3.302 (0.105)	3.020 (0.396)	2.859 (0.337)	4.622 (0.132)	5.090 (0.301)	4.600 (0.105)	4.507 (0.245)
Shoot C:N	10.836 (0.607)	10.853 (0.675)	11.625 (1.273)	10.352 (0.974)	9.519 (0.183)	10.152 (0.370)	9.660 (0.611)	9.267 (0.361)	14.238 (0.510)	13.034 (1.434)	15.483 (1.856)	15.395 (1.965)	8.430 (0.163)	8.428 (0.565)	9.343 (0.197)	8.969 (0.187)
Root biomass (g)	0.143 (0.021)	0.163 (0.036)	0.109 (0.026)	0.297 (0.161)	0.150 (0.072)	0.188 (0.036)	0.324 (0.119)	0.221 (0.087)	0.976 (0.115)	0.743 (0.075)	0.716 (0.113)	1.041 (0.148)	0.601 (0.081)	0.633 (0.062)	0.782 (0.136)	0.924 (0.051)
Root %C	37.140 (0.745)	31.930 (2.109)	35.209 (1.095)	34.862 (2.950)	37.575 (1.364)	34.600 (2.279)	30.609 (1.599)	31.365 (3.520)	27.643 (2.024)	32.988 (2.000)	30.626 (1.661)	29.687 (3.317)	28.457 (2.739)	31.720 (2.620)	29.408 (2.047)	29.808 (1.592)
Root %N	0.902 (0.029)	0.825 (0031)	0.864 (0.046)	0.889 (0.065)	0.967 (0.053)	0.933 (0.062)	0.756 (0.033)	0.804 (0.088)	0.822 (0.062)	0.906 (0.049)	0.829 (0.027)	0.799 (0.076)	0.990 (0.111)	1.050 (0.072)	0.986 (0.065)	0.971 (0.058)
Root C:N	41.359 (1.420)	38.651 (1.420)	41.143 (1.853)	39.377 (3.014)	38.979 (1.430)	37.294 (2.152)	40.457 (0.355)	38.938 (1.307)	33.879 (1.599)	36.615 (1.988)	36.884 (1.297)	36.870 (1.339)	29.101 (1.206)	30.069 (0.851)	29.894 (1.331)	30.786 (0.565)
Shoot: root ratio	1.504 (0.229)	2.056 (0.869)	2.461 (0.494)	1.556 (0.344)	1.572 (0.135)	1.116 (0.185)	1.171 (0.181)	2.421 (0.529)	0.631 (0.051)	0.823 (0.071	0.835 (0.142)	0.765 (0.082)	1.155 (0.249)	0.942 (0.062)	1.352 (0.436)	0.829 (0.077)

Table S3.4. Soil and microbial characteristics across species, CO_2, N and microbial treatments. For the microbial treatment, *REST*=the restricted microbial treatment (<53µm) and *INC*=the inclusive microbial treatment (>53µm). Means are reported with one standard error. Sample sizes vary slightly and are reported for different treatment combinations in Table S5. Microbial biomass values from chloroform fumigation (MB C and MB N) are in µg C/g soil and all PLFA and NLFA values are in nmol/g soil.

	Agropyron repens								***Bromus inermis***							
	AMBIENT		**+ CO_2**		**+N**		**+CO_2 +N**		**AMBIENT**		**+ CO_2**		**+N**		**+CO_2 +N**	
	REST	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*
Soil %C	0.617 (0.017)	0.568 (0.076)	0.570 (0.030)	0.660 (0.019)	0.680 (0.009)	0.733 (0.112)	0.626 (0.100)	0.666 (0.055)	0.571 (0.031)	0.592 (0.041)	0.623 (0.046)	0.570 (0.038)	0.705 (0.056)	0.759 (0.048)	0.735 (0.037)	0.659 (0.020)
Soil %N	0.052 (0.002)	0.043 (0.006)	0.049 (0.003)	0.052 (0.001)	0.056 (0.001)	0.064 (0.009)	0.045 (0.003)	0.055 (0.003)	0.047 (0.002)	0.048 (0.002)	0.051 (0.004)	0.047 (0.002)	0.060 (0.004)	0.063 (0.004)	0.060 (0.003)	0.057 (0.001)
Soil C:N	12.004 (0.315)	12.605 (0.442)	11.743 (0.325)	12.619 (0.340)	12.224 (0.091)	11.451 (0.362)	11.671 (0.718)	12.062 (0.431)	11.999 (0.281)	12.275 (0.379)	12.326 (0.280)	12.061 (0.442)	11.641 (0.193)	11.982 (0.222)	12.216 (0.271)	11.626 (0.207)
Soil moisture %	7.802 (0.549)	8.864 (0.842)	9.668 (0.790)	8.642 (0.993)	9.363 (0.095)	8.883 (1.181)	9.681 (1.491)	8.605 (1.060)	6.063 (0.436)	7.723 (0.599)	8.117 (0.482)	5.994 (0.679)	6.198 (0.856)	5.814 (0.520)	4.686 (1.038)	5.956 (0.453)
Soil pH	6.548 (0.050)	6.463 (0.062)	6.414 (0.072)	6.513 (0.041)	6.573 (0.127)	6.378 (0.133)	6.423 (0.173)	6.541 (0.124)	6.558 (0.118)	6.660 (0.061)	6.381 (0.085)	6.490 (0.126)	6.583 (0.115)	6.405 (0.118)	6.584 (0.086)	6.360 (0.153)
MB C	11.515 (2.191)	12.202 (3.081)	15.552 (1.571)	11.988 (2.583)	14.870 (1.912)	14.079 (2.499)	17.429 (1.011)	14.814 (1.518)	15.304 (0.977)	20.043 (6.029)	17.903 (3.358)	21.005 (8.802)	21.217 (1.572)	20.470 (3.042)	17.974 (3.251)	15.774 (1.493)
MB N	2.135 (0.650)	2.487 (0.211)	2.523 (0.369)	2.518 (0.237)	3.203 (1.080)	3.571 (0.364)	2.043 (0.280)	3.262 (1.035)	3.385 (0.484)	4.042 (0.437)	4.348 (0.739)	3.607 (1.013)	5.093 (0.902)	4.062 (1.000)	5.643 (1.221)	4.302 (0.449)
MB C:N	6.384 (1.171)	4.894 (1.052)	6.642 (1.019)	5.690 (0.412)	5.456 (1.221)	4.412 (0.785)	8.593 (0.858)	7.684 (2.395)	5.071 (0.841)	4.657 (0.811)	4.098 (0.335)	6.436 (1.499)	5.113 (1.166)	5.603 (0.587)	3.276 (0.202)	3.753 (0.331)
Total MB (PLFA)	92.609 (6.829)	69.508 (13.823)	65.861 (7.913)	80.311 (21.012)	72.459 (12.873)	81.632 (11.662)	65.157 (7.435)	89.411 (15.305)	69.664 (8.375)	35.560 (9.052)	80.914 (20.407)	54.943 (19.166)	91.355 (12.662)	79.516 (21.320)	89.674 (10.358)	57.949 (19.868)
16:1ω5c PLFA	4.638 (0.299)	3.377 (0.626)	3.585 (0.450)	4.150 (1.116)	3.631 (0.584)	3.944 (0.736)	3.146 (0.440)	4.116 (0.705)	2.542 (0.934)	1.163 (0.409)	3.765 (0.922)	2.194 (1.138)	3.804 (0.801)	3.082 (1.096)	3.955 (0.504)	0.541 (0.379)
16:1ω5c NLFA	13.389 (2.187)	8.573 (1.615)	11.883 (3.351)	9.697 (1.326)	8.682 (3.095)	12.174 (1.899)	8.091 (2.491)	7.865 (1.802)	8.518 (1.988)	5.749 (1.436)	14.567 (2.104)	8.687 (1.677)	12.743 (1.479)	6.285 (1.418)	9.135 (1.559)	11.676 (3.314)
18:1ω9c PLFA	7.501 (0.915)	4.179 (0.844)	4.801 (0.670)	5.695 (1.935)	5.259 (0.718)	5.250 (1.314)	3.136 (1.598)	5.312 (0.796)	5.265 (0.709)	1.998 (0.901)	7.229 (2.152)	3.996 (1.265)	5.695 (1.245)	5.614 (2.070)	6.146 (0.588)	3.174 (1.640)
18:1ω9c NLFA	14.960 (4.091)	9.147 (1.468)	17.665 (8.210)	10.598 (2.556)	9.449 (1.922)	14.535 (1.783)	8.623 (3.193)	6.914 (1.491)	10.498 (4.022)	8.823 (2.335)	17.465 (4.269)	6.668 (1.744)	11.016 (2.769)	5.525 (1.977)	12.956 (2.316)	13.220 (2.725)
18:2ω6,9c PLFA	3.610 (0.591)	2.688 (0.968)	2.686 (0.324)	2.364 (0.737)	1.732 (0.621)	2.632 (0.712)	2.134 (0.240)	2.617 (0.621)	1.705 (0.322)	0.764 (0.294)	2.909 (1.128)	2.389 (0.807)	3.394 (1.119)	2.935 (1.252)	2.896 (0.424)	1.449 (0.842)
18:2ω6,9c NLFA	19.556 (9.798)	13.179 (5.866)	27.940 (20.493)	8.425 (3.309)	8.962 (5.744)	18.242 (6.288)	7.276 (5.130)	6.240 (1.569)	10.548 (4.736)	6.486 (3.288)	10.344 (4.661)	8.091 (2.313)	11.424 (3.807)	9.755 (4.122)	13.203 (5.810)	18.167 (7.943)
15:0 iso PLFA	5.217 (0.390)	3.617 (0.668)	3.970 (0.455)	5.188 (0.950)	4.725 (0.535)	5.082 (0.612)	4.286 (0.342)	5.534 (0.890)	4.407 (0.573)	1.563 (0.594)	5.369 (1.288)	3.249 (1.059)	5.200 (0.492)	5.284 (1.585)	4.761 (0.536)	3.980 (1.537)
16:1ω7c PLFA	7.872 (0.452)	5.624 (1.250)	5.643 (0.564)	6.956 (1.693)	6.234 (0.906)	6.509 (0.770)	5.606 (0.045)	7.203 (1.364)	6.599 (0.643)	2.962 (1.103)	7.104 (1.749)	4.653 (1.573)	7.738 (0.976)	6.413 (1.374)	7.132 (0.947)	5.963 (2.392)

Table S3.5. Sample sizes for measurements of all variables across all species, CO_2, N and microbial treatment combinations. For the microbial treatment, *REST*=the restricted microbial treatment (<53µm) and *INC*=the inclusive microbial treatment (>53µm). Overall, nine out of 96 total planted samples did not germinate and there was one sample where the end member data produced an inaccurate model; all ten of these samples were excluded from all analyses. Additionally, certain variables have slightly altered sample sizes due to measurement issues that resulted in inaccurate data, which were excluded from the analysis; **a** two root 13C samples were outside range of QC; **b** two soil 13C samples were outside range of QC; **c** three microbial biomass N samples were excluded for being negative; **d** four root biomass samples were excluded due to inability to measure sample.

	Agropyron repens								***Bromus inermis***							
	AMBIENT		**+ CO_2**		**+N**		**+CO_2 +N**		**AMBIENT**		**+ CO_2**		**+N**		**+CO_2 +N**	
	REST	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*	*REST*	*INC*
Total biomass (g)[d]	5	5	6	3	2	5	3	6	6	6	6	5	6	6	6	6
Shoot biomass (g)	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Shoot %C	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Shoot %N	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Shoot C:N	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Root biomass (g)[d]	5	5	6	3	2	5	3	6	6	6	6	5	6	6	6	6
Root %C[a]	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
Root %N[a]	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
Root C:N[a]	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
Shoot: root ratio[d]	5	5	6	3	2	5	3	6	6	6	6	5	6	6	6	6
Soil %C	6	6	6	4	3	5	3	6	6	6	6	5	5	6	6	6
Soil %N	6	5	6	4	3	5	2	6	6	6	6	5	5	6	6	6
Soil C:N	6	5	6	4	3	5	2	6	6	6	6	5	5	6	6	6
Soil moisture	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
Soil pH	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
MB C	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
MB N	6	6	6	3	3	4	3	5	6	6	6	5	6	6	6	6
MB C:N	6	6	6	3	3	4	3	5	6	6	6	5	6	6	6	6
Total MB (PLFA)	5	5	6	4	2	5	3	6	5	6	6	5	6	6	6	6
All PLFA/NLFAs	5	5	6	4	2	5	3	6	5	6	6	5	6	6	6	6
CO_2 $\delta^{13}C$	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Shoot $\delta^{13}C$	6	6	6	4	3	5	3	6	6	6	6	5	6	6	6	6
Root $\delta^{13}C$[a]	6	5	6	4	3	5	2	6	6	6	6	5	6	6	6	6
Soil $\delta^{13}C$[b]	6	6	6	4	3	5	3	6	6	6	6	5	5	6	6	6

Figure S3.1. Whole-plant chamber used for CO_2 analysis.

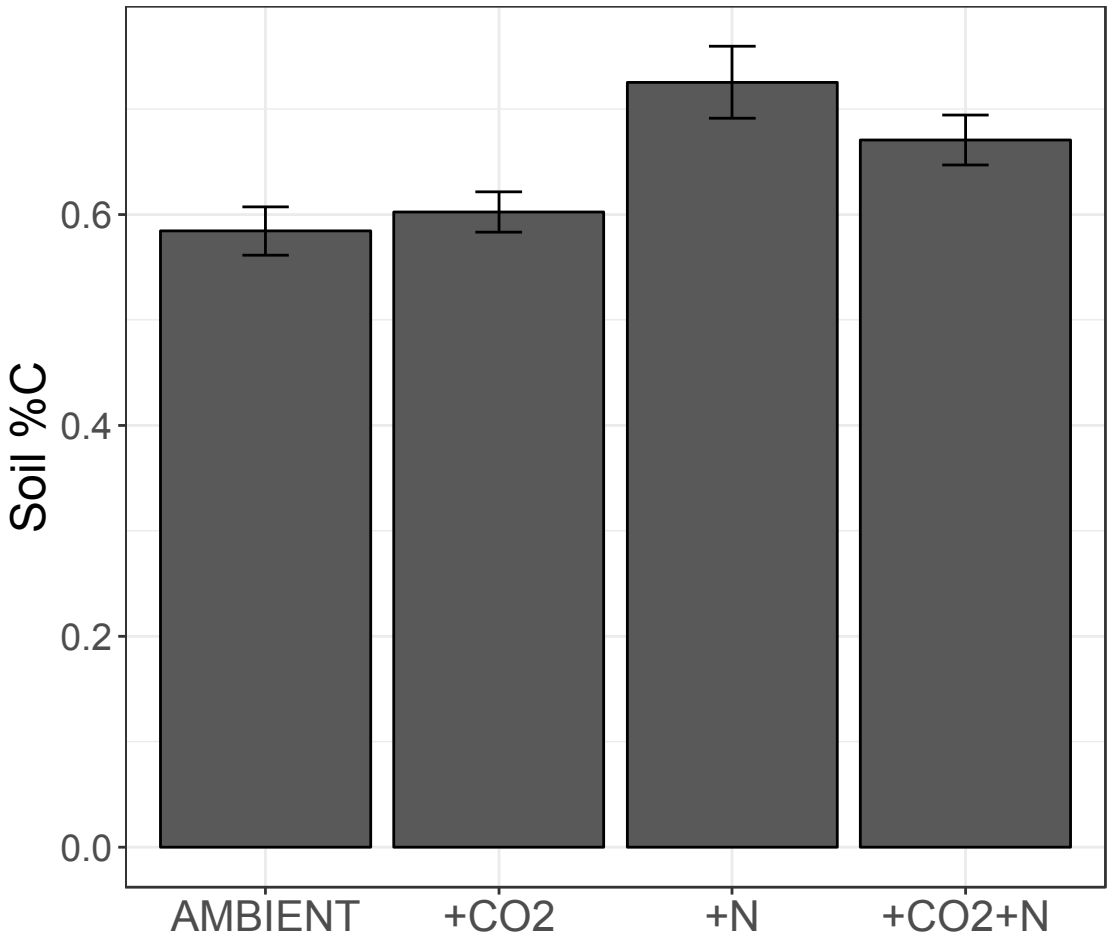

Figure S3.2. Soil %C across CO_2 and N treatments.

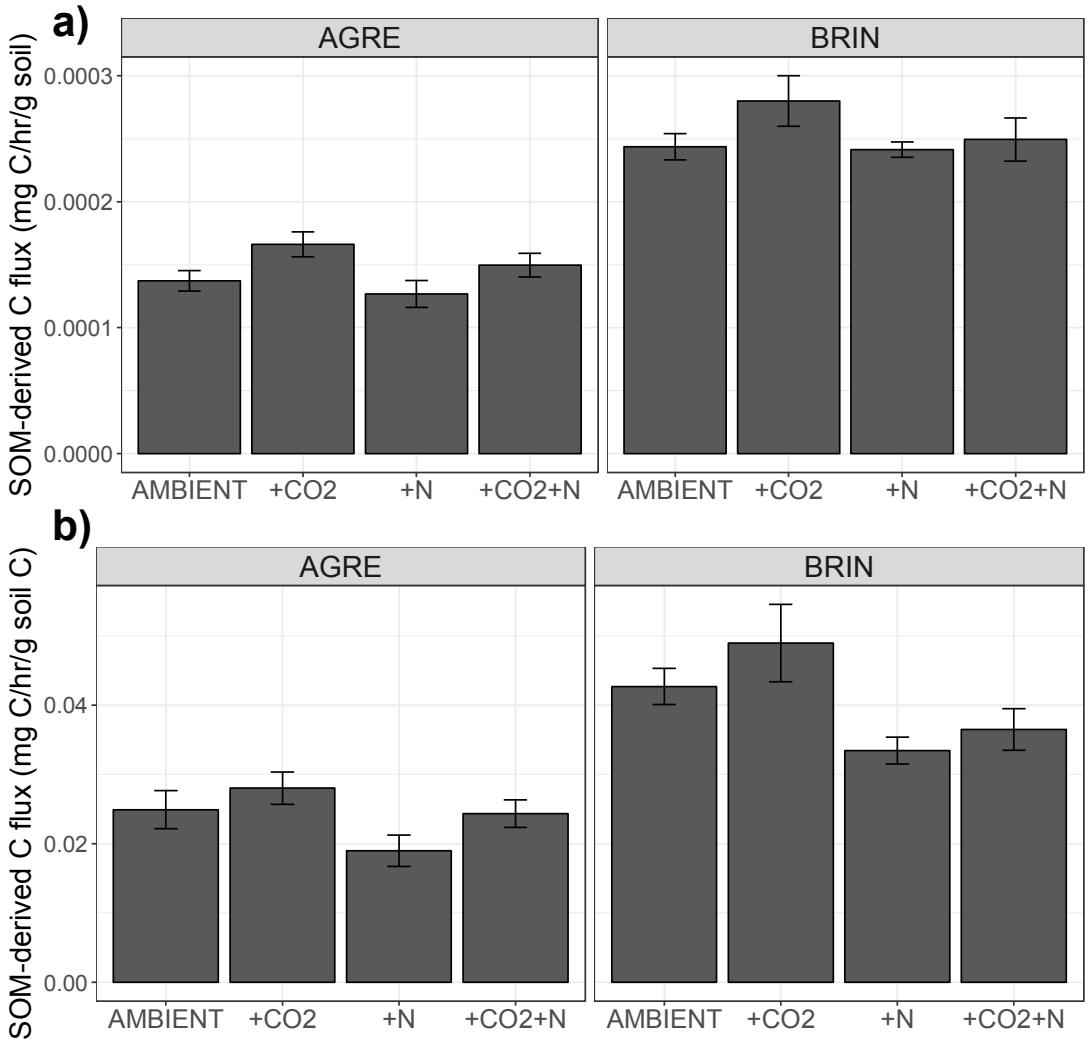

Figure S3.3. The amount of SOM-derived C flux across plant species, CO_2, and N treatments, expressed as **a)** mg C / hr / g soil and **b)** mg C / hr / g soil C. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*.

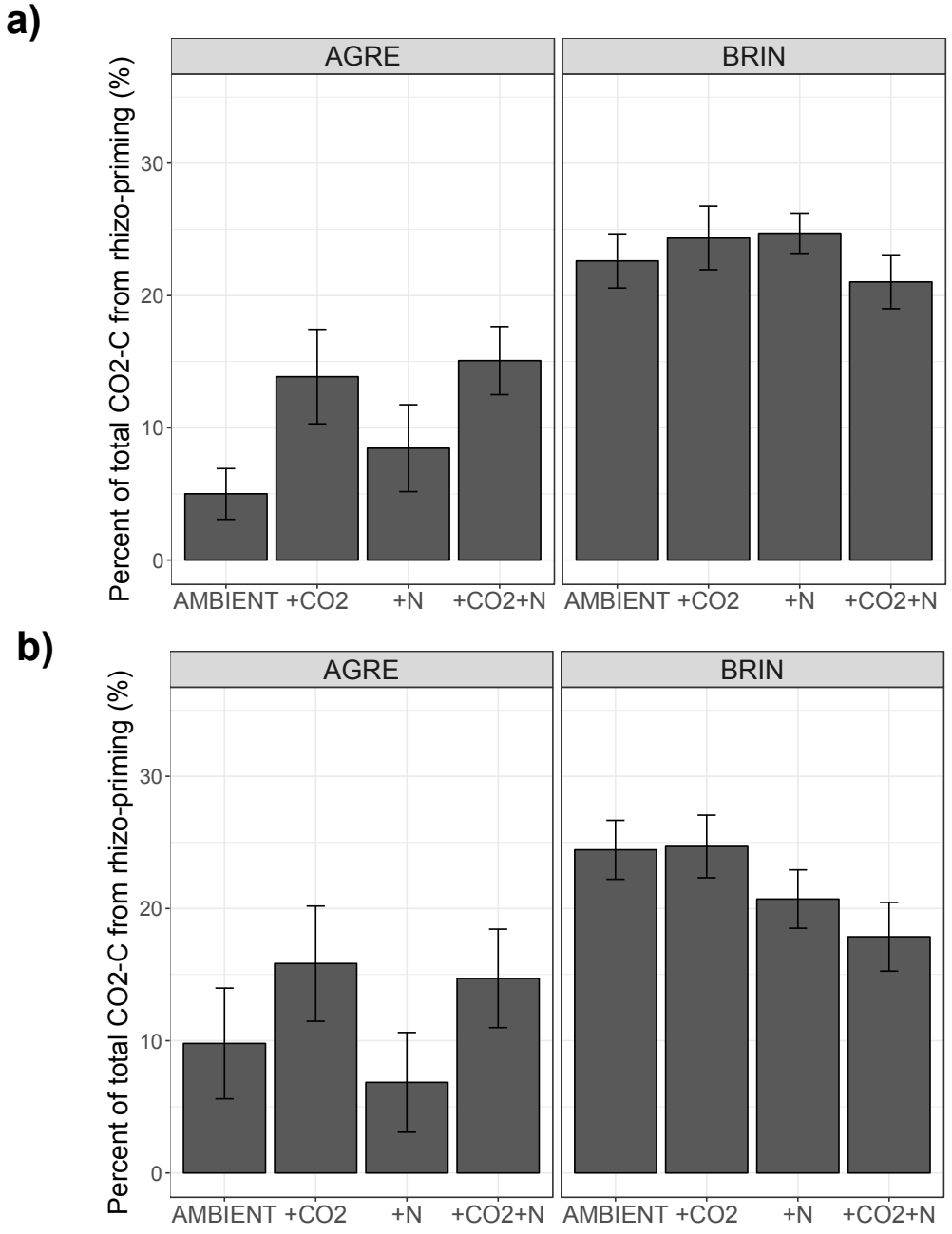

Figure S3.4. The percent of the total CO_2-C flux from primed SOM-C across CO_2, N, and plant species, calculated from flux amounts expressed as **a)** mg C / hr / g soil and **b)** mg C / hr / g soil C. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*.

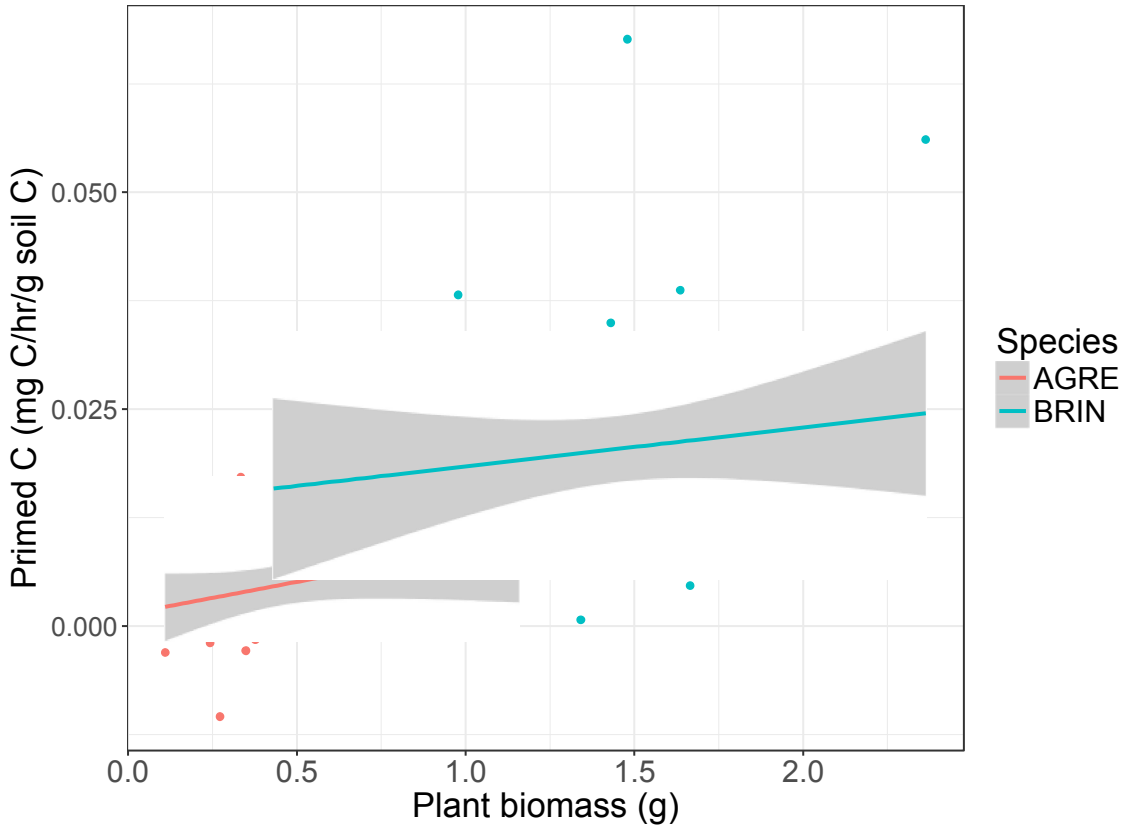

Figure S3.5. Relationship between the amount of C from rhizosphere priming of soil organic matter decomposition (mg C / hr / g soil) and total plant biomass (shoots and roots) in the two different species. AGRE = *Agropyron repens*; BRIN = *Bromus inermis*.

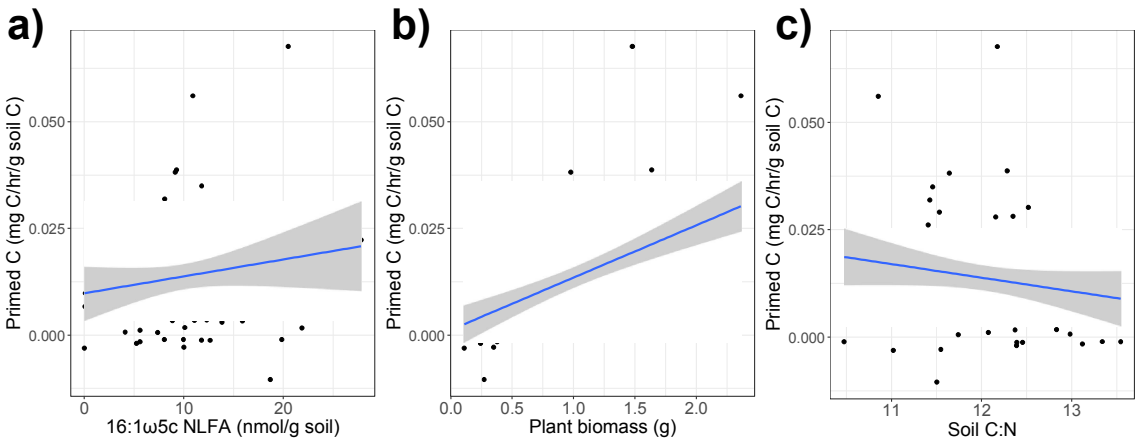

Figure S3.6. Relationships between the amount of C from rhizopriming (mg C / hr / g soil) and three covariates included in models: a) 16:1ω5c neutral lipid abundance, b) total plant biomass, and c) soil C:N ratio.

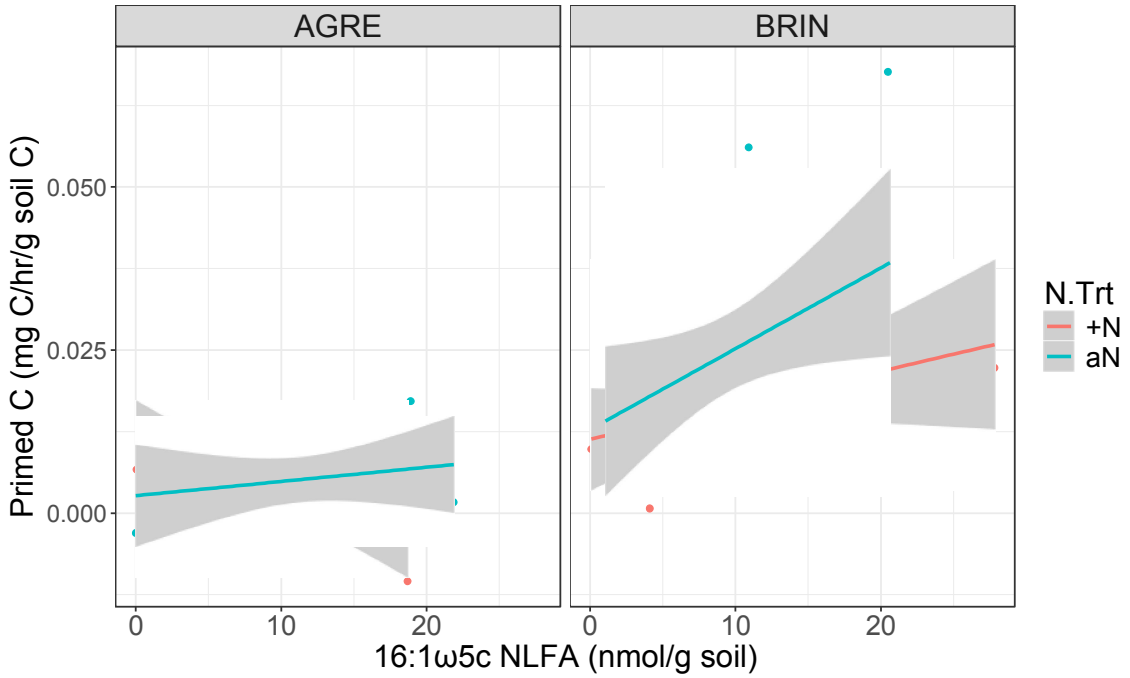

Figure S3.7. Relationships between the amount of SOM-C released from rhizosphere priming (mg C / hr / g soil) and 16:1ω5c neutral lipid abundance by species and N treatment.